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Isolation and Characterisation of Halogenated and Bioactive Compounds from *Thalassospira* sp. and *Alteromonas* sp.

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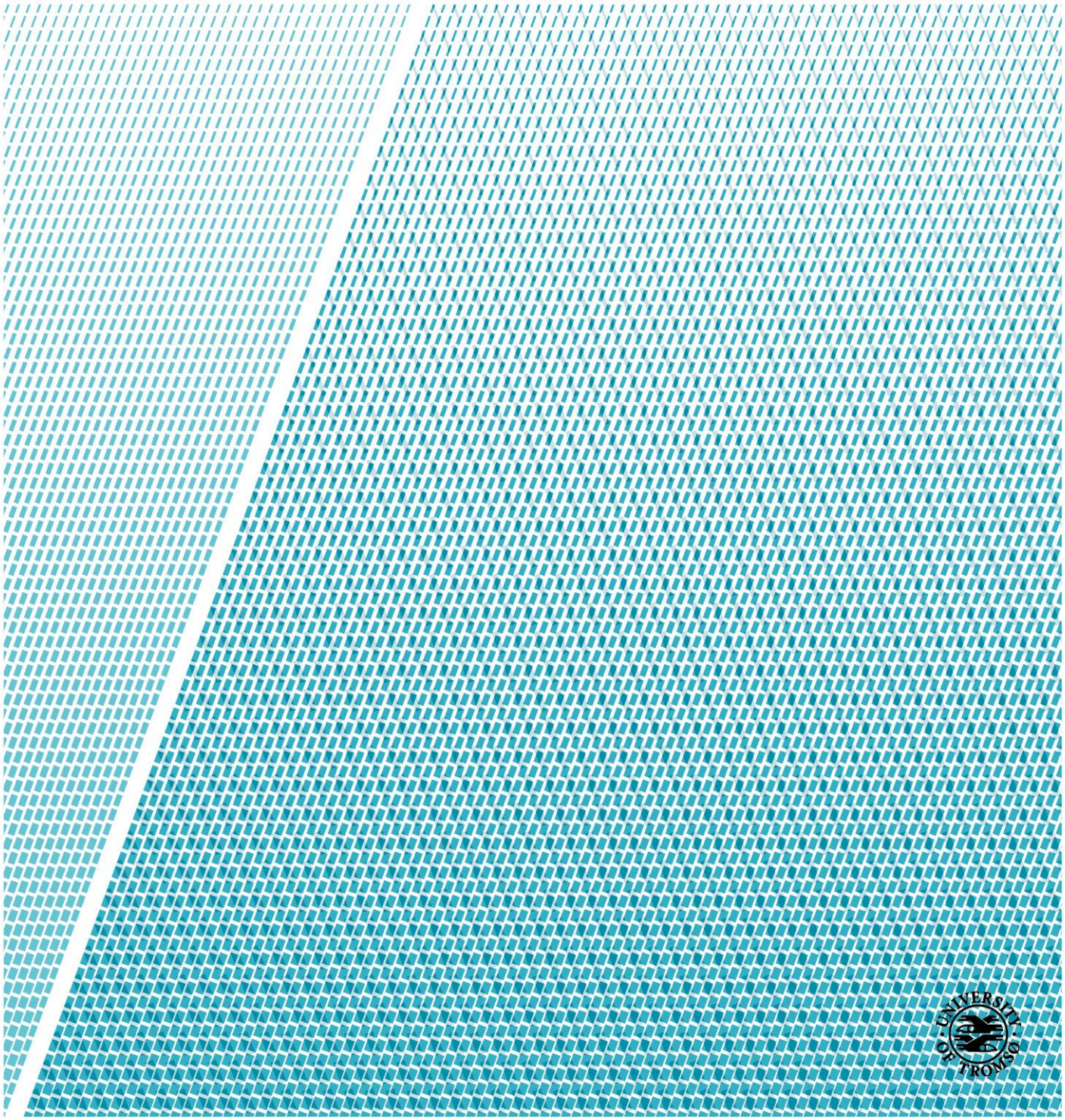
Master's thesis in Pharmacy (May 2019)



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اريد ان اعرب عن خالص امتناني وتقديري لجهود المشرف وعائلتي واصدقائي على رسالتي والذي حفزني للوصول الى النهايه

Tromsø, May 2019

Nadia Ghazi Ali

Abstract

Nature has served as source for several bioactive compounds with great chemical diversity, and the application of natural products in drug development has been a remarkable success. The bioprospecting efforts have previously been concentrated on terrestrial organisms, whereas organisms living in marine environments were left largely underutilised. Therefore, marine organisms have moved into focus as improvements in technologies have made the marine environments more easily accessible (1).

Bacterial natural products have contributed with a large part of the bioactive compounds that lead to drugs, and the biggest portion of the marketed antibiotics are bacterial products or their derivatives (2). Marine bacteria are not yet investigated to the same extend as their terrestrial counterparts. Therefore, they are considered a promising source for novel bioactive metabolites for drug development, which has been a trigger for the work carried out in this thesis. In this thesis, three Arctic marine bacteria of the genus *Thalassospira*, *Alteromonas* and *Pseudomonas* were investigated. The bacterial strains were cultivated, extracted and pre-fractionated. The fractions were screened for antibacterial and anticancer activity, and active fractions were re-fractionated. The bioactive re-fractionated extracts were dereplicated to investigate if the compounds suspected of being active, were novel or had been previously reported. Compounds assumed to be bioactive, as well as those with interesting chemical properties were isolated and characterised. One bioactive compound was isolated from the bacterial strain *Thalassospira* sp. The compound was active in both antibacterial and anticancer assays, and its structure remains to be elucidated. A di-halogenated compound was also isolated from this strain, it was however found to be chemically unstable. Moreover, three compounds were isolated from the bacterial strain *Alteromonas* sp. that remain to be structure elucidated and screened for bioactivity.

Table of Contents

Abbreviations

1D	One-Dimensional
2D	Two-Dimensional
ACE	Angiotensin Converting Enzyme
AM	Alteromonas
BHI	Brain Heart Infusion broth
COSY	COrrelation SpectroscopY
DAD	Diode Array Detector
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DWP	Deep-Well Plate
ESI	Electrospray Ionisation
FA	Formic Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GNPS	Global Natural Products Social molecular networking
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-Performance Liquid Chromatography
HR	High Resolution
HSQC	Heteronuclear Single Quantum Coherence
LC	Liquid Chromatography
m/z	Mass-to-Charge
MH	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
MMNP	Marine Microbial Natural Product
MNP	Marine Natural Product
MRSA	Methicillin-Resistant Staphylococcus aureus
MS	Mass Spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H- tetrazolium salt
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser SpectroscopY
NP	Natural Product
PM	Pseudomonas
QToF	Quadrupole Time-of-Flight
ROESY	Rotating-frame Overhauser SpectroscoPY
RP	Reversed Phase
RPMI	Roswell Park Meomorial Institue Medium
SPE	Solid Phase Extraction
TOCSY	TOtal Correlated SpectroscopY
TS	Thalassospira
UHPLC	Ultra-High-Performance Liquid Chromatography
UV	Ultraviolet

1.0 Introduction

1.1 Natural products

Natural products (NPs) are chemical compounds synthesised by living organisms such as plants, animals and microorganisms (3, 4). The term ‘’NPs’’ refers often to secondary metabolites, because they are predominantly not biosynthesised by the general metabolic pathways as primary metabolites, and they are not required for normal growth, development or reproduction of an organism (4, 5). Secondary metabolites are produced by organisms in response to needs and challenges of the natural environment, and they provide the producing organisms with benefits in various ways, for instance by improving nutrient availability or by defending against predation and other environmental stressors (e.g., pigments and compatible solutes). These metabolites are often exclusive for a set of species within a phylogenetic group. Secondary metabolites usually exhibit some sort of biological activity as they have effects on living organisms or living tissues, and these are referred to as bioactive secondary metabolites (6-8). The focus of this work is metabolites produced during cultivation of marine bacteria.

There is an urgent need for new therapeutic agents, particularly novel antibiotics, to fight diseases and drug-resistant pathogens that poses threats to the public health. The exploration and development of new therapeutic agents from NPs have played a central role throughout the development of modern medicine as these products present several advantages when compared with synthetic compounds, such as high chemical diversity, binding efficiency and tendency to interact with high specificity and potency with biological targets, that makes them favourable lead compounds for drug discovery (5, 9, 10). This has resulted in a high variety and number of therapeutic compounds being discovered in nature, most of which stem from terrestrial plants or microbes. Some examples are the cholesterol-lowering drugs Lovastatin and Simvastatin, which are derived from the fungus *Aspergillus terrus*, the angiotensin-converting enzyme (ACE) inhibitors Captopril and Enalapril, which are derived from peptides isolated from the venom of the Brazillian pit viper *Bothrops jaraca,* Amoxicillin (antibiotic) which is derived from *Penicillium fungi*, Phenoxymethyl-penicillin (antibiotic) which is derived from *Penicillium chrysogenum* and Taxol (anticancer drug) which is derived from *Taxus brevifolia* (11-15). The chemical structures of the mentioned compounds can be seen in Fig 1.

NPs typically contain more complex scaffolds in contrast with synthetic drugs which are generally observed as flat, rigid molecules with a high degree of aromatic character. Among

other things NPs contain a larger number of ring systems and chiral centres, making them sterically more complex structures. In addition, NPs contain a lower number of nitrogens, halogen and sulphur atoms, whereas the content of oxygen is higher compared with synthetic drugs. NPs also have on average higher molecular weights, lower hydrophobicity and increased polarity compared to synthetic drugs. These structural features enable NPs to provide highly selective and specific biological activities. For instance, increased content of chiral centres in a molecule has been associated with enhanced binding selectivity (15).

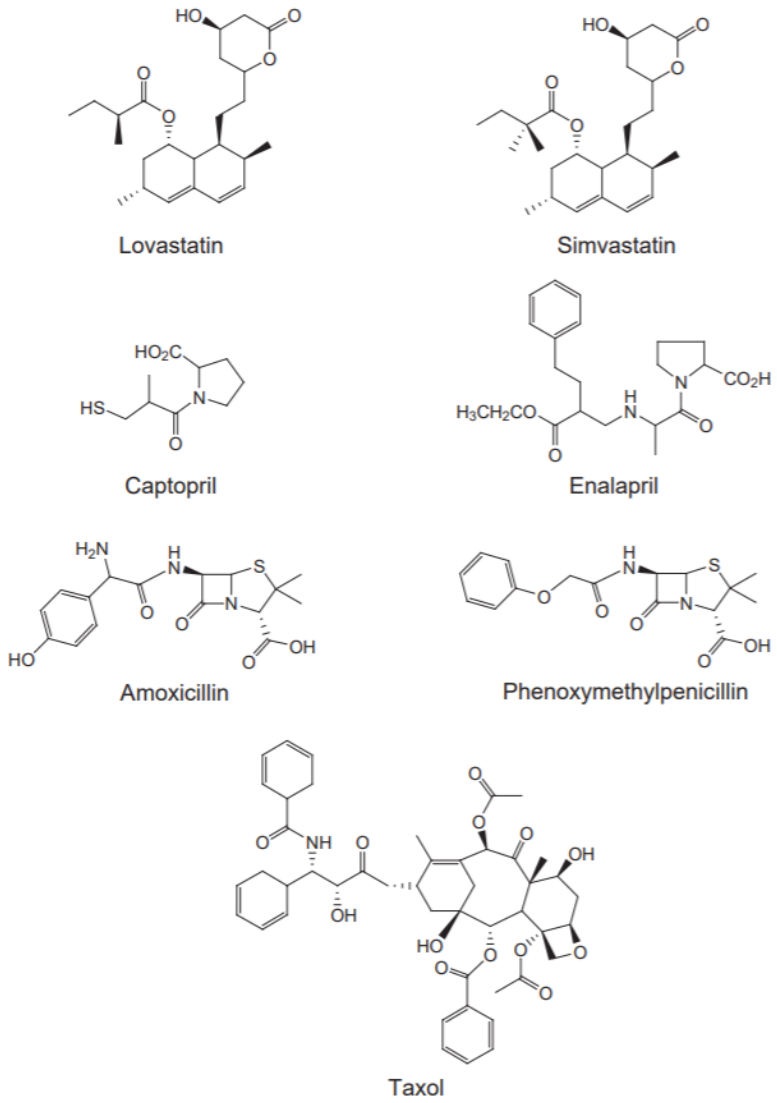

Figure 1: Chemical structure of some drugs derived from NPs (12). Lovastatin and simvastatin are derived from the fungus Aspergillus terreus (13). Captopril and Enalapril are derived from peptides isolated from the venom of the Brazillian pit viper Bothrops jararaca (14). Amoxicillin and Phenoxymethylpenicillin are derived from the fungus Penicillium fungi and Penicillium chrysogenum, respectively. Taxol is derived from the bark of the Pacific Yew tree (Taxus brevifolia) (16).

1.2 The marine environment

Approximately three quarters of the earth's surface is covered by seas and oceans which represent an enormous resource for the discovery of potential therapeutic compounds. The marine environment is a relatively untapped reservoir of novel NPs in contrast to the terrestrial environment. More than 15,000 structurally diverse NPs with a wide array of bioactivities have been discovered from the marine environment since the 1970s (9). There has been a growing interest in searching the marine environment for novel bioactive compounds during the last decades, due to its enormous variety in habitats with high diversities of organisms (17). The marine environment has certain unique characteristics compared to the terrestrial environment, such as high salinity, high hydrostatic pressure, high or low temperatures, high salt concentrations and low concentrations of organic matter, making the organisms living in marine environments metabolically and physiologically different from those living in terrestrial environments (18, 19). Nevertheless, it represents a widely unexplored authority for isolation of novel microbes like bacteria, fungi, actinomycetes, microalgae-cyanobacteria and diatoms, that are potent synthesisers of bioactive secondary metabolites (20). However, the marine environment is considered capable to provide new lead compounds against pathogenic microbes that are developing resistance to existing pharmaceuticals (21).

1.3 Marine bioprospecting

Marine bioprospecting is the process of research and development of novel bioactive compounds based on marine organisms, including microorganisms like bacteria, fungi and viruses as well as macroorganisms such as sea plants, shellfish and fish. The principle of marine bioprospecting is to search for components, compounds or genes that can be included as components in products or processes. Suitable fields of application may include medicine, processing industries, oil and gas, food, feed and biofuels. The marine organisms may stem from the sea, the coast, the fjord, the seabed or oil reservoirs beneath the seabed (22). This thesis will focus on the use of marine natural products (MNPs) as lead compounds with potential for further development into antibiotics and drugs for anticancer treatment.

Marine organisms represent abundant sources of structurally diverse bioactive compounds with various and often potent biological activities, many of which belong to new chemical classes not found in terrestrial NPs (23, 24). The first antibiotic of marine microbial origin,

pentabromopseudilin, isolated from the bacteria *Pseudomonas bromoutilis* in 1966, has a unique highly brominated chemical structure not seen in the products of terrestrial origin (Fig. 2) (18).

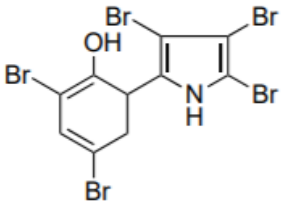

Figure 2: Chemical structure of an antibiotic produced by the marine bacteria Pseudomonas bromoutilis (18).

1.4 Marine natural products

Marine organisms are known to produce a wide array of secondary metabolites to ensure their survival, persistence and competitiveness in diverse and hostile habitats, some of which can be used as lead compounds in the development of new pharmaceuticals (5, 25, 26). They are in general rich sources of pharmacologically active compounds with great chemical diversity and complexity (27). The chemical diversity associated with compounds derived from marine sources is boundless, differing from simple linear peptides and fatty acids to complex alkaloids, terpenes and polyketides (28). These structurally distinct molecules have been reported to have various bioactive properties such as anticancer, anti-inflammatory, anti-proliferative, cytotoxic and antibiotic properties (20, 28). Some of these compounds possess novel mechanisms of action. For example, the anticancer agent trabectedin (Yondelis®; Fig. 3) derived from the marine tunicate *Ecteinascidia turbinate,* shows a unique and complex mechanism of action causing DNA damage and distinctively interferes with the transcription-coupled nucleotide repair system by binding to the DNA minor groove, resulting in apoptosis in cancer cells that show highly accelerated gene expression compared with normal cells (29).

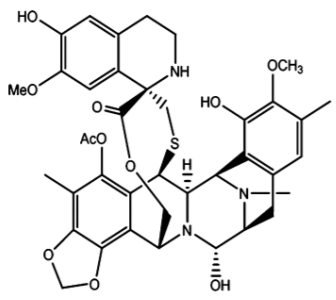

Figure 3: Chemical structure of trabectedin (30).

One of the first MNPs to enter clinical trials as an anticancer agent was Didemnin B (Fig. 4), isolated from the tunicate *Tridemnum solidum* (31, 32). However, the compound did not make it to the market as it turned out to be too toxic for safe use (33). Despite the fact that Didemnin B was not successfully developed into a drug, since then a plethora of drug candidates have been isolated from marine organisms, and interest has slowly shifted from terrestrial to marine bioresources due to the adventitious properties afforded by the MNPs (31, 34). Marine-derived compounds show higher incidence of significant bioactivity compared to those of terrestrial origin (29, 35). For instance, almost 1% of the tested marine samples in a National Cancer Institute preclinical cytotoxicity screen showed anticancer potential, whereas only 0.1% of the tested terrestrial samples showed anticancer potential (29). Certain pharmaceuticals have been developed from lead compounds of marine origin, such as the anticancer drug Cytarabine (Ara-C®) and the antiviral drug Vidarabine (Ara-A®), which are nucleosides derived from the Caribbean sponges *Cryptotheca crypta* and *Tethya crypta*, respectively. These were the first marine-derived drugs approved by the Food and Drug Administration (FDA) in the United States, in 1969 and 1976, respectively. Another pharmaceutical of marine origin is the peptide Ziconotide (Prialt®), derived from the tropical marine cone snail *Conus magus*, which produces the peptide to inactivate its prey. Prialt® was approved by FDA in the United States in 2004 for treatment of chronic pain (29, 36, 37). The chemical structures of the mentioned compounds can be seen in Fig. 4.

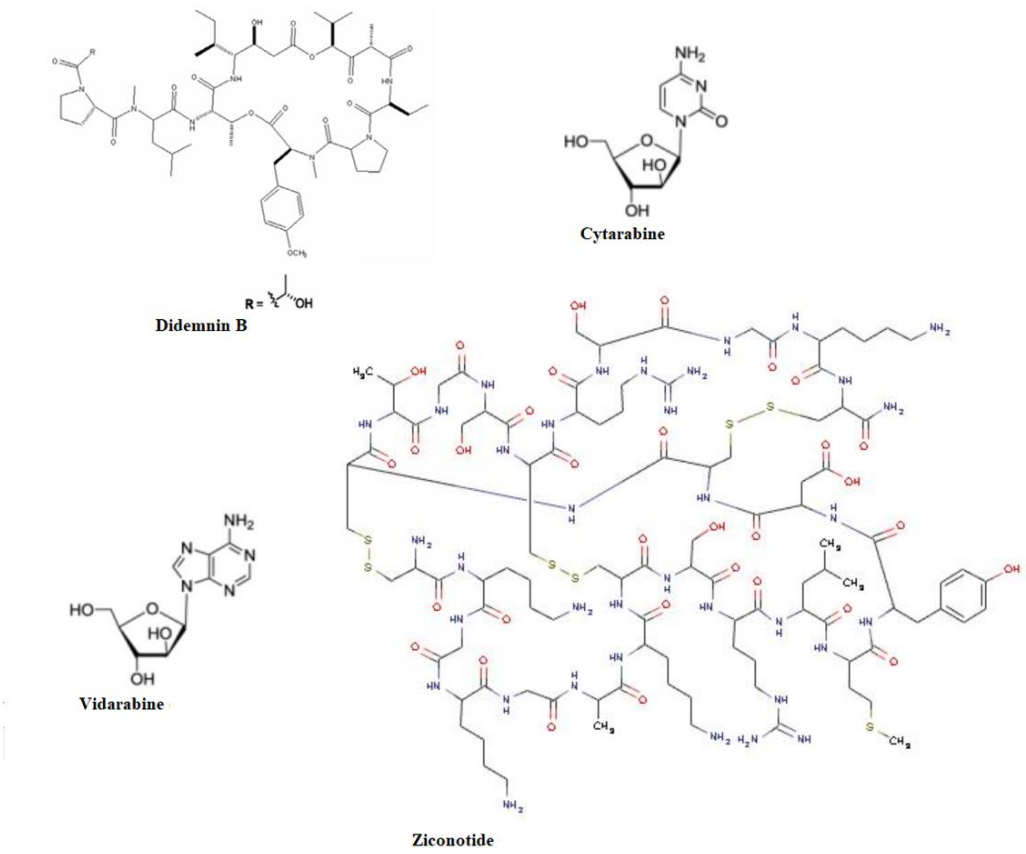

Figure 4: The chemical structure of the marine derived compounds Didemnin B (31), Cytarabine (38), Vidarabine (38) and Ziconotide (39).

1.5 Marine microbial natural products

Marine microorganisms are currently of considerable interest in discovery of novel pharmaceutical lead compounds, as some of the compounds isolated from marine invertebrates are suspected of being produced by symbiotic microorganisms rather than the invertebrate itself. (25, 40, 41). For example, the potential marine anticancer compound Bryostatin 1 (Fig. 5) which is currently under Phase II clinical trials, was initially considered to be synthesised by *Bryozoa*. However, recently it has been confirmed that it is actually synthesised by the bacterial symbiont, *Candidatus Endobugula sertula* (20). Another example is that the antifungal compound Theopalauamide (Fig. 5), isolated from the sponge *Theonella swinhoei*, was also found to be associated with fractions from the δ-Proteobacterium *Entotheonella palauensis* within extracts of *Theonella swinhoei* (41).

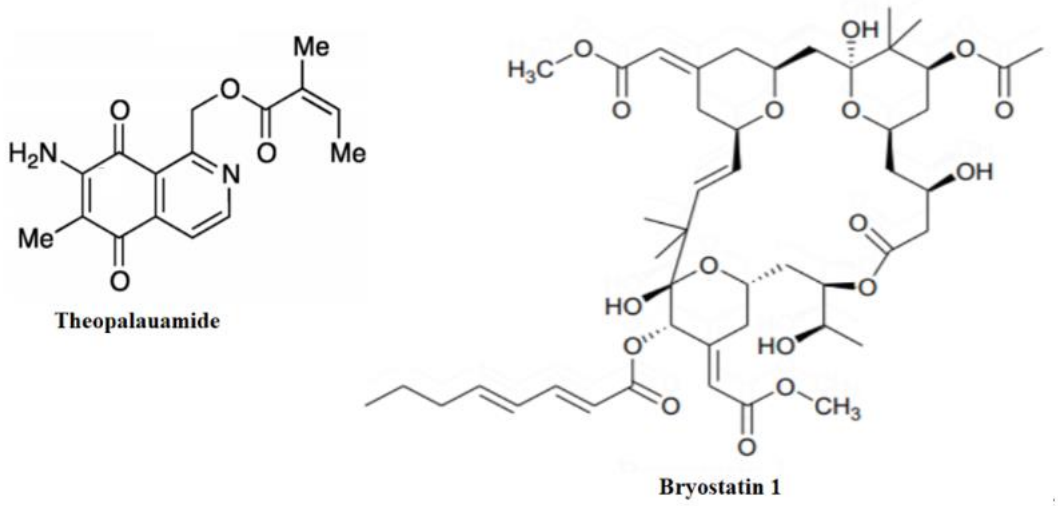

Figure 5: The chemical structure of the potential anticancer compound Bryostatin 1 (20) and the antifungal compound Theopalauamide (41).

In recent years, there has been growing awareness that the most productive source of unique NPs is actually microorganisms, including actinomycetes, cyanobacteria and microalgae such as dinoflagellates (36). However, it is estimated that less than 5% of marine microbial species are identified yet, indicating that the microbial diversity of oceans is still insufficiently understood (42). It is also estimated that less than 0.1% of all the microorganisms existing in the oceans have been explored till now and among them, only a small fraction have been cultivated in the laboratory due to the lack of knowledge concerning nutritional needs and environmental requirements of these organisms (43). Nevertheless, marine microbial natural products (MMNPs) are now promoted to be pharmaceutically important lead compounds, due to improving knowledge of the groups of bacteria and other microbial sources existing in the oceans, and the rapid development of novel technologies and tools, such as advances in bioinformatics and analytical techniques (20, 44, 45). Several potential pharmaceuticals from marine microbial sources with various bioactivities are at different stages of clinical testing currently (46). For instance, the proteasome inhibitor Salinosporamide A (NPI-0052: Fig. 6) isolated from the marine bacterium *Salinispora tropica* is in Phase I clinical trials for the treatment of multiple myeloma, whereas the peptide Plinabulin (NPI-2358: Fig. 6) developed from the fungal diketopiperazine halimide, derived from a marine fungus *Aspergillus* sp. is currently in Phase III clinical trials for the treatment of non-small cell lung cancer. Other examples are the promising anticancer compounds Tasidoton (ILX-651: Fig. 6) and Solidotin (TZT 1027: Fig. 6) which are both isolated from marine bacteria. They are currently under Phase II and Phase III clinical trials, respectively (20, 47). Fig. 7 gives an overview of the

numbers and the proportion of bioactive/non-bioactive compounds discovered from various marine organisms between 1985 and 2012, illustrating that the highest proportion of bioactive compounds were discovered from marine bacteria and actinomycetes (48). MMNPs represent a promising source for drug discovery, in particular for antibacterial and anticancer drugs (49).

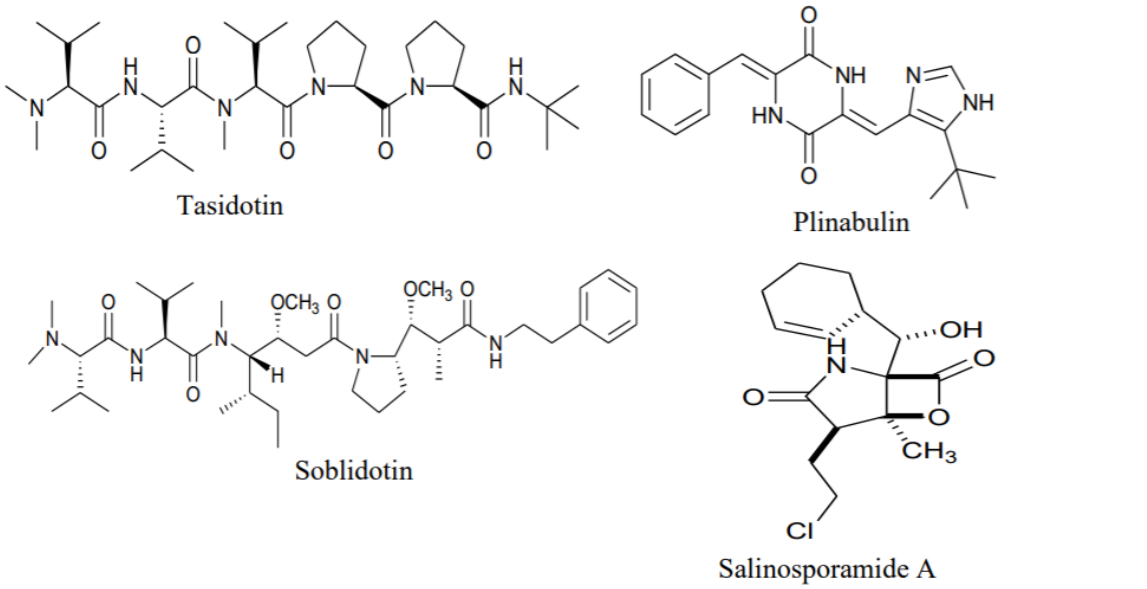

Figure 6: The chemical structures of Tasidotin, Plinabulin, Soblidotin and Salinosporamide A (20).

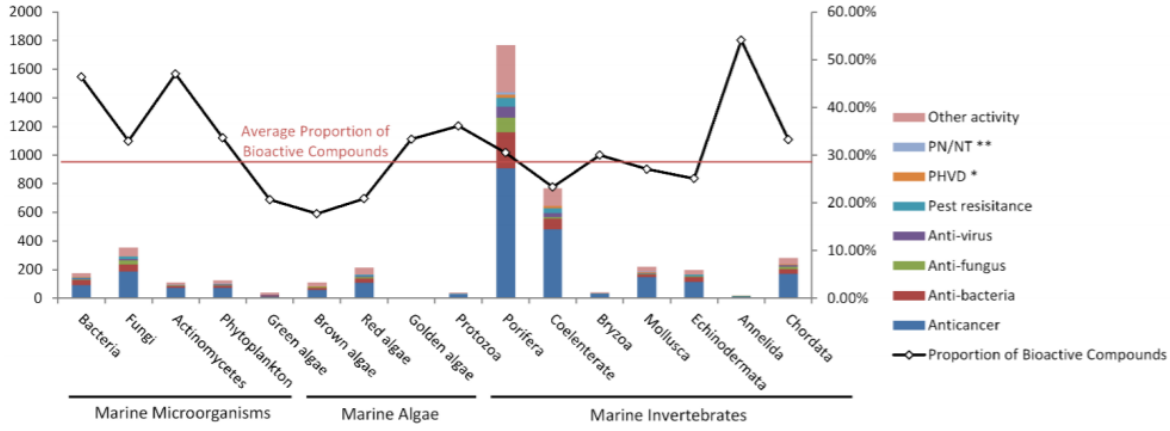

Figure 7: Number and proportion of bioactive and non-bioactive compounds discovered from marine organisms between 1985 and 2012 (48).

1.6 Bacteria

1.6.1 The bacterial growth curve

The bacterial growth curve represents changes in quantity of a bacterial population over time in a batch culture, which is a closed system. Bacteria are not able to grow exponentially endlessly in such a system, since parameters necessary for growth such as pH, temperature, concentrations of nutrients, metabolic products and oxygen, change during the different growth phases of a batch culture. After a period of time, a negative feedback between growth rate and one or several of these parameters in the culture will lead to a steady number of cells and will eventually result in a decrease in cell number (50, 51).

The bacterial growth curve consists of four distinct phases, which are the lag phase, the exponential (log) phase, the stationary phase and the death phase. The lag phase is the first phase observed in a batch system. In this phase, the bacteria are inoculated into the culture medium and they start producing necessary primary metabolites such as enzymes needed for their growth in the particular medium (50). Subsequently after the lag phase comes the exponential phase, where the bacteria rely on available resources to maintain exponential growth. This phase continues until nutrients are reduced or toxic products increase and other environmental parameters like temperature and pH value change, at which the rate of exponential growth slows, and the bacteria enter the stationary phase. In the stationary phase, the bacterial growth reaches a steady state where there is neither an increased nor a reduced number of cells. Bacteria synthesise secondary metabolites through this phase of the growth cycle (52). In the end, the bacteria enter the death phase due to unfavourable conditions. Fig. 8A presents the distinct phases of bacterial growth curve in culture medium (53). Temperature and other environmental parameters affect the growth rate of bacteria. Bacteria grow at different temperatures depending on the conditions in the natural environment of the organism, and they can be classified based on the temperature range at which they can grow (Fig. 8B). Bacteria classified as psychrophiles grow at low

temperatures, mesophiles grow at moderate temperatures while those classified as thermophiles and hyperthermophiles grow at high temperatures (54).

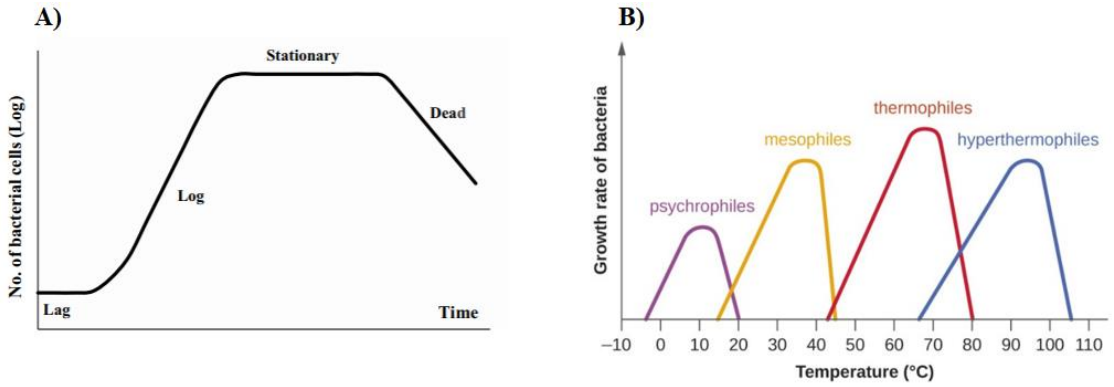

Figure 8: A) The distinct phases of bacterial growth curve in culture medium (53). B) Growth rate of bacteria as a function of temperature (55).

1.6.2 Marine bacteria

Marine bacteria may live in unique and extreme habitats with exclusive circumstances of pH, temperature, pressure, oxygen, light, nutrients and salinity, which is particularly rich in chlorine and bromine. They are able to sense instantly, adapt, respond to their extreme habitats and contend for defence and survival by synthesising attractive unique NPs. Recent advances in microbial genomics such as genome sequencing, microbial ecology and metagenomics have certainly indicated that the biosynthetic potential of NPs in bacteria is much higher than previously acknowledged (56). Marine bacteria thrive in the ocean's surface waters as well as in the lower and immeasurable depths (42). Bacteria that grow in the marine environment are generally thermophiles, psychrophiles, mesophiles, halophiles (thriving at high salinity), alkalophiles (thriving at high pH), piezophiles (adapted to high pressure) or polyextremophiles (adapted simultaneously to several stresses) (19). Thermophilic bacteria grow close to the occasional hot thermal vents where hot magma spills out on the seabed (57). However, marine bacteria are generally psychrophiles that grow at low temperatures since the average temperature of the oceans is below 5 °C (58). Psychrophilic bacteria synthesise enzymes that are optimally active at low temperatures, and lose activity at moderate temperatures (59).

Marine bacteria grow under different conditions, as their nutritional needs and environmental requirements vary, and this must be taken into account when cultivating bacteria in the laboratory. Many of the marine bacteria require seawater or 3% sodium chloride to freshwater in the medium for growth (60). Whereas other marine bacteria require artificial brackish water

with various carbon substrates such as agarose, starch, laminarin, xylan, chitin and glucose for growth. It is essential to mimic the natural environment for bacteria to grow and produce secondary metabolites in culture medium (9).

1.6.2.1 *Alteromonas* sp.

Alteromonas is a genus consisting of Gram-negative, rod shaped, aerobic, moderately halophilic bacteria with polar flagellum motility. Members of the genus *Alteromonas* occur globally in the surface as well as the deep seawater, and have been isolated from temperate or tropical seas and thus are generally mesophiles growing between 10 and 45 °C. Some of the species are also psychrophiles that have been isolated from habitats featuring cold temperatures such as *Alteromonas stellipolaris* and *Alteromonas litorea* (61). Furthermore, the members of this genus are considered typical fast growing ‘’blooms’’ that can be easily isolated and cultivated in the laboratory (62, 63). However, they depend on the presence of organic nutrients in the marine setting for growth (64).

1.6.2.2 *Pseudomonas* sp.

Bacteria of the genus *Pseudomonas* are one of the most well-studied species. They occur in most environments and considered to be among the most successful phyla of bacteria, based on their low nutritional requirements as well as great metabolic diversity, which allows them to utilise various organic compounds as a source of both carbon and energy. Members of the genus *Pseudomonas* synthesise a wide variety of secondary metabolites, which are essential for their own survival. Hence, the bacteria of the genus *Pseudomonas* represent an interesting alternative for production of various potential beneficial compounds (65). This is a genus consisting of Gram-negative, aerobic, motile by one or several polar flagella, non-spore-forming straight or slightly curved rod-shaped bacteria (66).

1.6.2.3 Thalassospira sp.

The genus *Thalassospira* consists of Gram-negative, motile, vibrio- or spiral-shaped, halotolerant and chemoheterotrophic bacteria. Bacteria of this genus are widely distributed in various marine environments, such as seawater, sediment and halobios from every ocean and sea (67). Some of the spices are psychrophilic or mesophilic that grow at different temperatures between 4 and 40 °C with various salt concentrations (68-70). It has been observed that some

bacteria within the genus *Thalassospira* are able to synthesise thalassospiramides, beta-galactosidase and biosurfactants (67).

2.0 Aim of this work

The overall aim of this work was to isolate and characterise compounds with antibiotic and anticancer activity potentially produced by the marine bacteria *Thalassospira* sp. (M10B738), *Alteromonas* sp. (M09W202) and *Pseudomonas* sp. (M10B807).

The key objectives of the thesis were to:

1. Screen the fractions from bacterial cultures for antibacterial and anticancer activity.
2. Dereplicate the bioactive fractions to identify the active compounds.
3. Isolate and elucidate the structure of compounds that appear likely to be novel after dereplication.

3.0 Workflow and background

3.1 Workflow

This work was conducted at Marbio, and the practical work performed through the project was based on their commonly used methods. Marbio is a high throughput analytical platform for isolation and screening of secondary metabolites produced by marine organisms. Fig. 9 gives an overview of the practical work performed through the project, and the background for each of the steps will be described in the subsequent sections.

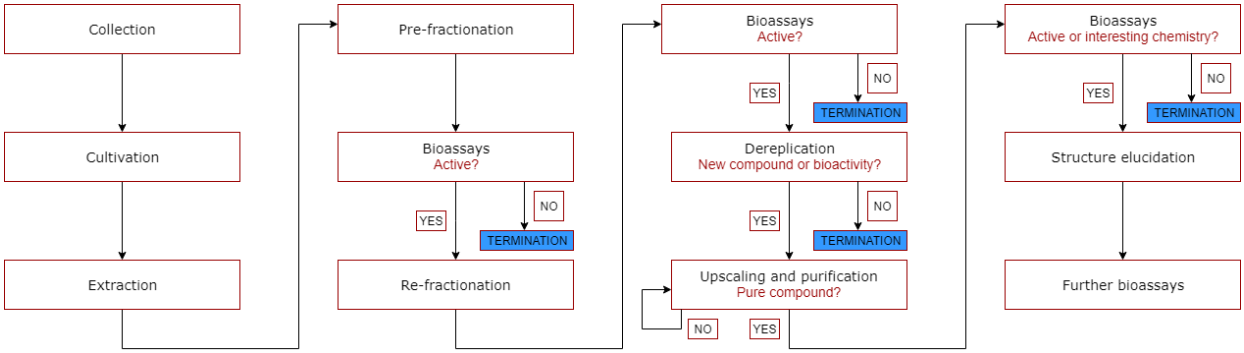

Figure 9: Workflow for the work performed through this project. The figure is made with inspiration from the reference (71).

3.2 Background

3.2.1 Cultivation and extraction

In this work process, three Arctic marine bacteria collected by Marbank, were cultivated in different culture media depending on the environmental and nutritional growth requirements of the bacteria. Production of secondary metabolites is controlled by the choice of media, temperature, aeration and duration of the fermentation (72). After cultivation, secondary metabolites synthesised by the bacteria must be extracted from the culture for further separation and characterisation. Solid-phase extraction (SPE) was performed using Diaion® HP-20 and methanol, for extraction of secondary metabolites from the bacterial culture. Diaion® HP-20 is a non-polar copolymer styrene-divinylbenzene adsorbent resin used to adsorb hydrophobic compounds such as antibiotics and biomolecules. This resin is recommended for the adsorption of solute molecules with molecular weights lower than 20-30 kilodaltons (73).

3.2.2 Pre-fractionation of the extracts

Crude extracts are a complex mixture of media components, salts, primary and secondary metabolites in different concentrations, consequently they are less suitable for instant bioactivity screening. It is therefore often required to desalt and fractionate the extract into fractions containing compounds of similar characteristics, such as polarity or molecular size (74). Pre-fractionation of extracts to reduce chemical complexity increases the hit rates due to a higher concentration of the active compound(s) (36). However, it is not appropriate to generate many fractions, as it may spread the active compound over so many fractions that the fractions containing this compound in low concentrations might not be detected or not show any activity in bioassays (75). In this project, the extracts were fractionated into six flash fractions.

The most frequently used pre-fractionation technique to separate compounds in a mixture, is liquid chromatography (LC) such as high-performance liquid chromatography (HPLC) or flash chromatography. The sample is loaded onto a column where compounds in the sample are separated by a gradient of water and an organic solvent, and the retention times of the different compounds depend on their interaction with the stationary phase and the mobile phase (75). In this project, flash chromatography was applied for the pre-fractionation of the extracts. Flash

chromatography can be applied to separate compounds by size, polarity, charge or affinity depending on the column material and mobile phases used. Advantages of flash chromatography is high loading capacity and low costs. However, it is not expected to provide the same resolution or reproducibility as HPLC. This is nonetheless not needed in pre-fractionation, since a positive hit will nominate the fraction for isolation of the bioactive compounds using preparative HPLC Mass Spectrometry (HPLC-MS). The stationary phase utilised in this project was Diaion® HP-20SS, a synthetic styrene-divinylbenzene polymer adsorbent used for the separation of hydrophobic compounds as well as biomolecules, and the compounds were separated by polarity (76).

3.2.3 Bioactivity screening of flash fractions

Bioassays are performed to evaluate the bioactivities of compounds in a sample, by, for example, observing their effects on cell lines or pathogenic bacteria (77). These assays are essential for the discovery of new bioactive lead compounds (78). The process of discovering a novel lead compound against a selected target for a specific disease normally involves high throughput screening (HTS), where a large number of compounds are screened for activity against the target (79). In this project, the flash fractions were screened for possible antibacterial and anticancer activities.

3.2.3.1 Antibacterial activity screening

Antibiotics are substances used in the treatment and prevention of infections caused by bacteria. The need for novel antibiotics is widespread due to the fact that most pathogenic bacteria that cause infections have developed resistance to existing antibiotics. Antibiotic resistance is considered to be one of the biggest threats to global health, as many infections such as pneumonia and tuberculosis, are becoming more difficult to treat since the antibiotics used in their treatment turn into less effective substances (80). Therefore, there has been an increasing interest in investigating and developing novel and effective antibiotics from various sources, and more emphasis has been placed on antibacterial activity screening. Commonly used bioassays are e.g. disk-diffusion, well diffusion and broth or agar dilution (81).

In this project, the antibacterial activity screening of the fractions was performed using a minimum inhibitory concentration (MIC) assay, which is a method used to determine the lowest concentration of an antibiotic needed to inhibit the growth of bacteria. MIC assays are

commonly used in discovering antibacterial secondary metabolites in both crude extracts and fractions (82). The antibacterial activity of the secondary metabolites was assessed against six known human pathogens: *Staphylococcus aureus* (Gram-positive), *Enterococcus faecalis* (Gram-positive), *Pseudomonas aeruginosa* (Gram-negative), *Escherichia coli* (Gram-negative), *Streptococcus* type B (Gram-positive) and Methicillin-resistant *Staphylococcus aureus* (Gram-positive).

3.2.3.2 Anticancer activity screening

Cancer is accountable for an estimated 9.6 million deaths in 2018, making it the second leading cause of mortality worldwide (83). It is characterised by uncontrolled proliferation of cells due to dysfunction of regulatory signalling pathways that are normally under tight control, and can spread quickly into surrounding tissues as well as organs and cause damage (84). The most common types of cancer are lung, breast, colorectal, prostate, skin and stomach cancer (83).

Increasing recurrence of cancer and severe adverse effects of chemotherapeutic agents reduces the clinical efficacy of a wide range of anticancer drugs currently used. Hence, there is a constant need to develop alternative or synergistic anticancer drugs with minimal adverse effects (85).

In this project, an Aqueous One Solution Cell Proliferation assay was applied to screen the fractions for anticancer activity. This is a colorimetric method used to determine if the test fractions inhibit cell proliferation or display direct cytotoxic effects that eventually lead to cell death, by measuring cell viability (86, 87). The assay contains a tetrazolium salt, called MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (87). Metabolically active cells are able to reduce this salt into a dark blue coloured formazan product, and the quantity of formazan that is produced is directly proportional to the level of viable cells. Formazan absorbs light at 490 nm and can be determined spectrophotometrically (86, 87).

3.2.4 Re-fractionation of active flash fractions

The active flash fractions were re-fractionated in order to isolate the bioactive compounds using preparative HPLC-MS (Fig. 10). There is a wide range of compounds in the active flash fraction. Hence, it is necessary to fractionate it further in order to identify the active compounds. Reversed phase HPLC-MS (RP-HPLC-MS) used in the project is particularly well suited to

deal with the quantities and polarity of compounds developed for pharmaceutical interest. Thus, it is widely used for the purification of NPs from complex mixtures (88). RP columns such as C18 are used to separate compounds within a wide range of polarities and molecular masses. These columns have a hydrophobic stationary phase and consequently the chromatography is based on hydrophobic interactions between the compounds and the stationary phase (74). The mobile phase, in which the compounds are eluted, is split by a flow splitter to the fraction collector and to the ultraviolet (UV) and MS detectors after separation in the column. The main advantage of preparative RP-HPLC-MS is that it is selective, and hence allows for efficient separation of compounds with similar masses (88).

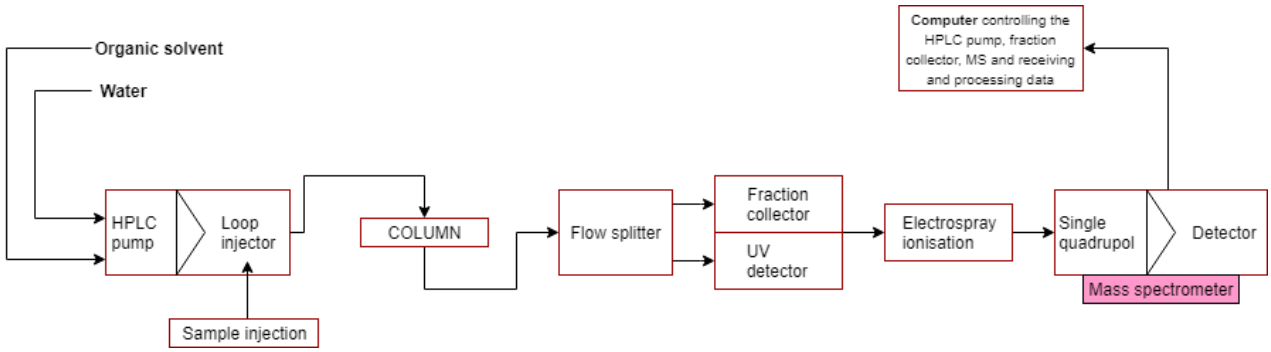

Figure 10: Schematic illustration of the preparative-HPLC-MS system used in re-fractionation and for compound isolation in this project. The figure is made with inspiration from the reference (71).

3.2.5 Bioactivity screening of subfractions

After re-fractionation, the 40 subfractions obtained were screened for antibacterial and anticancer activity as described previously.

3.2.6 Dereplication

Dereplication is the process of identification of already known bioactive compounds using a combination of analytical and spectroscopic methods. This is an essential part of the NP drug discovery process to avoid further work on active compounds that are previously known and focus effort on the discovery of structurally novel compounds (89). Hence, the dereplication process has the purpose of making productive use of often limited resources (4).

The fractions with confirmed bioactivity were selected for dereplication. The dereplication process was performed using Ultra-High-Performance Liquid Chromatography in combination with Quadrupole-Time-of-Flight MS (UHPLC-QToF-MS). The instrument consists of five

components that can be seen in Fig. 11. The UHPLC unit is separating the compounds within the extract chemically and directing them into the electron ionisation source where the molecules are ionised (90). ESI may be applied to generate both positive and negative ions (91). The ionised analytes are then electrostatically propelled into the mass analyser where they are separated according to their mass-to-charge (*m/z*) ratios (92). For detection, Quadrupole-Time-of-Flight (Q-ToF) was applied as it provides high mass accuracy and high mass resolution, and thus separates compounds with similar masses efficiently (93). This mass analyser is a variant of a triple quadrupole where the third quadrupole is replaced by a ToF analyser. In addition to the molecular mass of analytes, the fragment data generated by the collision cell allows a better identification of compounds by comparing molecular fragments.

Figure 11: Illustration of the five components of a MS system: sample inlet, ionisation source, mass analyser, detector and the data system.

High resolution MS (HR-MS) in combination with liquid chromatography is a preferred method of dereplication in NP drug discovery, since this combination is considered both sensitive and suitable for analysing complex mixtures (94). The major advantage of this method is that it can provide accurate mass which can be used to calculate the elemental composition, which in turn can be used to search databases like MarinLit, Dictionary of Natural Products, ChemSpider and SciFinder to identify known compounds (94, 95). Nevertheless, only compounds that are identical to those in the databases will be identified using this approach, whereas those that are similar but non-identical to existing compounds will not be recognised (94). Moreover, the inherent disadvantage of this approach is that database searches provide a list of NPs of which molecular mass are similar to the observed one, where the compounds in the list are often false positives (96). However, fragmentation patterns of compounds can be used to assist in their identification. The fragmentation data can be utilised to search MS/MS libraries such as Global Natural Products Social molecular networking (GNPS) (94).

3.2.7 Upscaling and purification

Some compounds of interest may be challenging to identify, either due to low concentrations or interference of other substances which prevent identification with sufficient certainty, thus it may be necessary to upscale and purify these compounds in order to identify their chemical structures correctly. Purified compounds in amounts ranging from one to a few milligrams are

required for identification and characterisation of unknown compounds in NP chemistry (97, 98). In this work process, MS guided preparative HPLC (Fig. 10) was utilised to purify and isolate the compounds which were found to be promising candidates.

3.2.8 Bioactivity confirmation of the isolated compounds

The promising isolated candidates from which enough material was obtained, were screened for antibacterial activity against the bacterial strain *Streptococcus* type B, and for anticancer activity against the cell line A2058, in order to confirm their activity. The bioactive compounds were submitted for structure elucidation by Nuclear magnetic resonance (NMR) spectroscopy.

3.2.9 Structure elucidation using NMR

NMR remains together with MS the first choice in organic structure elucidation. This approach is based on the principle that many atomic nuclei spin about an axis and thus have magnetic properties that can be utilised to generate NMR spectra with chemical information. In an NMR spectrometer the nuclei in a sample is subjected to radiofrequency radiation from a range of 60 to 1000 MHz in the presence of an external magnetic field, making the nuclei in the sample absorb the energy and consequently spin from the low energy to the high energy state. The frequency required for absorption of energy is characteristic of the type of nucleus (e.g., ^{1}H or ^{13}C) and depends on their chemical environment as well as the strength of the external magnetic field, which is measured as an NMR signal (99). Several NMR techniques that yield various chemical information are required to propose the structure of compounds, including one-dimensional (1D) such as ^{1}H DEPT and ^{13}C NMR spectra as well as two-dimensional (2D) such as COSY (COrrelation SpectroscopY), HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) NMR spectra. ^{1}H and ^{13}C NMR spectra yield information about the number and chemical environment of protons and carbons in the chemical structure (100, 101). COSY NMR spectrum provides information about the coupling between nuclei (102), whereas HSQS and HMBC provide information about the correlation between two different nuclei separated by one bond and correlations over multiple bonds, respectively (103). 2D NMR techniques are utilised to determine the chemical structure of complex compounds. In addition, the MS data can provide an elemental composition and further information that may aid to solve the structure. The great advantage of NMR in NP chemistry is that the method is non-destructive for the sample (as for example in MS or bioassays) and the sample can be recovered by almost 100% after taking up the spectra.

3.2.10 Bioactivity screening of target compounds

The target compounds should be screened for bioactivity in various assays in order to characterise their full activities.

4.0 Materials and methods

4.1 Sampling and storage of bacteria

Materials

Table 1: Equipment used in sampling and storage.

Equipment	Equipment identification	Supplier
Alteromonas sp.	M09W202	Marbank, Institute of Marine Research (Norway)
Pseudomonas sp.	M10B807	Marbank, Institute of Marine Research (Norway)
Thalassospira sp.	M10B738	Marbank, Institute of Marine Research (Norway)
Difco Marine Broth	279110	Becton, Dickinson and Company (New Jersey, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (St. Louis, MO, USA)
Glycerol	G5516	Sigma-Aldrich (St. Louis, MO, USA)
Filtered sea water	-	Norwegian College of Fishery Science, UiT (Norway)
MilliQ Ultrapure water	-	Merck KGaA (Germany)
Sanyo Labo Autoclave	-	Sanyo Electric Co. (Japan)
Stuart Heat Stir SB162	-	Stuart Equipment (UK)

Three Arctic marine bacterial strains from the Marbank collection were used in this project; M09W202 from the genus *Alteromonas* sp., M10B807 from the genus *Pseudomonas* sp. and M10B738 from the genus *Thalassospira* sp. The bacteria will be shortened AM for *Alteromonas*, PM for *Pseudomonas* and TS for *Thalassospira* throughout the thesis. AM was isolated from seawater and collected the 22th of May 2009 in the Barents Sea in Norway with the Global Positioning System (GPS) coordinates of 75°38.400000N and 29°43.442561E. PM was isolated from a halibut (*Hippoglossus hippoglossus*) and collected the 16th of April 2010 in the Norwegian Sea with the GPS coordinates of 67°31.800000N and 13°21.963380E. TS was isolated from a sea-mouse (*Echinocardum cordatum*) and collected the 16th of April 2010 in the Norwegian Sea with the GPS coordinates of 68°30.000000N and 15°0.279511E. The bacteria were classified to genus level based on 16S rDNA sequence analysis by Marbank.

The bacterial strains were stored in FMAP (diluted Marine Agar-Peptone) medium with 30% glycerol at -80 °C. The composition of the medium is listed in Table 4. Glycerol was added to the medium, and the solution was autoclaved at 120 °C for 30 minutes. The bacterial stock was prepared by stroking the bacteria from freeze stock strains stored at -80 °C on a FMAP-agar plate, and the plate was incubated for 3-7 days at 10 °C. A single colony was selected and

transferred to 5 mL FMAP medium and incubated for 2-3 days at 10 °C with shaking at 300 rpm. 500 µL of the culture was transferred to cryo tubes with 1 mL of FMAP medium and stored at -80 °C.

4.2 Preparation of cultivation media

Materials

Table 2: Equipment used in the preparation of cultivation media.

Equipment	Equpiment identification	Supplier
D-Mannitol	63560	Sigma-Aldrich (St. Louis, MO, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (St. Louis, MO, USA)
Malt extract	70167	Sigma-Aldrich (St. Louis, MO, USA)
Yeast extract	Y1625	Sigma-Aldrich (St. Louis, MO, USA)
D-glucose (dextrose)	D9434	Sigma-Aldrich (St. Louis, MO, USA)
Casaminoacid (casein hydrolase)	22090	Sigma-Aldrich (St. Louis, MO, USA)
Filtered seawater	-	Norwegian College of Fishery Science, UiT (Norway)
MilliQ water	-	Merck KGaA (Germany)
Sanyo Labo Autoclave	-	Sanyo Electric Co. (Japan)
Infors HT Multitron Pro	-	Infors HT (Switzerland)

Method

The bacterial strains M09W202, M10B807 and M10B738 were cultivated in three different media (Table 3). The composition of each medium is listed in Table 4. All media were sterilised by autoclaving at 121 °C for 30 minutes, and they were cooled to room temperature before the bacterial strains were inoculated. The media with bacterial strains were incubated at 10 °C and shaken at 130 rpm in the shaking incubator for 9, 12 and 32 days, respectively (Table 3).

Table 3: Cutivation parameters used for the three bacterial strains, including the medium used for each of the strain, temperature, shaking speed and time for the cultivation.

Strain Nr.:	Media	Temperature and shaking	Cultivation time (days)
M09W202 (X0845)	DSGC	10 °C, 130 rpm	9
M10B807 (X0834B)	DM19	10 °C, 130 rpm	32
M10B738 (X0811B)	DVR1	10 °C, 130 rpm	12

Table 4: Contents of the media used for cultivation of the bacterial strains and the medium (FMAP) in which the bacterial strains were stored.

Media	Chemicals	Amount
DSGC	D-glucose (dextrose) Casaminoacid (casein hydrolase) Filtered seawater	3.6 g (0.4 %) 2.7 g (0.3 %) 900 mL
DM19	D-Mannitol Peptone from casein, enzymatic digest MilliQ water Filtered sea water	18 g 18 g 450 mL 450 mL
DVR1	Malt extract Peptone from casein, enzymatic digest Yeast extract MilliQ water Filtered sea water	6 g 10 g 6 g 450 mL 450 mL
FMAP	Difco Marine broth Peptone MilliQ water Filtered sea water	15 g 5 g 700 mL 300 mL

4.3 Extraction of the bacterial cultures

Materials

Table 5: Equipment used in the extraction of secondary metabolites from the bacterial cultures.

Equipment	Equipment identification	Supplier
Methanol (MeOH)	20864	VWR International S.A.S (France)
MilliQ water	-	Merck KGaA (Germany)
Cheesecloth filter, fine mesh	-	Dansk Hjemmeproduktion (Denmark)
Whatman® qualitative filter paper, grade 3	1003-090	GE Healthcare Life Sciences (UK)
Biofuge Pico Heraeus centrifuge	-	Thermo Fisher Scientifie (Massachusetts, USA)
Rotary Evaporator (Rotavapor)	Laborota	Heidolph Instruments GmbH & Co. (Germany)
Diaion® HP-20	99611	Sigma-Aldrich (St. Louis, MO, USA)
Universal Shaker SM 30	-	Edmund Buhler GmbH (Germany)

Method

Diaion HP-20 resin was added to the media 3-4 days before the end of the culture for extraction of the secreted secondary metabolites. 12 g of resin Diaion HP-20 was weighed out in 100 mL Erlenmeyer flasks. The resin was activated by soaking it in 100% methanol for minimum 30 minutes, methanol was then removed carefully and replaced with MilliQ water for 15 minutes. MilliQ water was removed before resin was added to the cultures.

A sample of 400 µL was taken from the cultures for DNA test prior to extraction (section 4.3.1 ‘’Treatment of DNA sample of bacterial culture’’).

The culture medium was removed by pouring it over the filter with resin beads remaining in the culture flask, and the resin was washed with 200 mL MilliQ water and filtered through cheesecloth mesh. 150 mL of methanol was added to the flasks with resin. The flasks were subsequently incubated at room temperature for 1 hour with gentle shaking for extraction of the secreted secondary metabolites from the resin. After incubation, the extract was filtered through a Whatman filter paper. The resin was extacted two times with methanol as described. A sample of 400 µL was taken from the extracts for a MS analysis, and the MS sample was stored at –20 °C. The extracts were dried under reduced pressure at 40 °C using the rotavapor and stored at –20 °C until further use.

4.3.1 Treatment of DNA samples of bacterial cultures

400 µL of the culture was transferred to an Eppendorf tube and centrifuged for 3 minutes at 18928 relative centrifugal force (rcf). The supernatant was decanted, and the pellet washed with 1 mL MilliQ water. The sample was centrifuged for 3 minutes at 18928 rcf again, and the supernatant decanted. The Eppendorf tube with the pellet was labelled with bacterial strain and stored at -20 °C for identity check of the cultures.

4.4 Flash chromatography of the extracts

Materials

Table 6: Equipment used in the pre-fractionation of extracts.

Equipment	Equipment identification	Supplier
Methanol	20864	VWR International S.A.S (France)
Aceton	34850	Sigma-Aldrich, (St. Louis, MO, USA)
Diaion® HP-20SS	13615	Sigma-Aldrich (St. Louis, MO, USA)
MilliQ water	-	Merck KGaA (Germany)
Biotage® SP4 Flash Purification System	-	Biotage (Sweden)
Biotage® SNAP Cartridge KP-Sil (10 g)	FSK0-1107-0010	Biotage (Sweden)
Rotary Evaporator (Rotavapor)	Laborota	Heidolph Instruments GmbH & Co. (Germany)
Büchi Syncore Polyvap	-	Büchi (Switzerland)

Method

4.4.1 Preparation of the extracts before flash fractionation

The extracts were weighed and dissolved in 20 mL of 90% methanol before adding Diaion® HP-20ss column material. 1.5 g Diaion® HP-20ss resin beads were added for each gram of extract. Subsequently, the mixture was dried under reduced pressure at 40 °C using the rotavapor.

4.4.2 Flash fractionation of the extracts

The sample was added to a prepacked SNAP column (see section 4.4.3 ‘’Preparation of SNAP columns for flash fractionation’’) when it was completely dry and run on the Flash Purification system with MilliQ water, methanol and acetone mobile phases using the gradient presented in Table 7. The flow rate was 20 mL/minute and each fraction consisted of 80 mL. From pre-fractionation, six fractions (F1-F6) divided in 27 flash tubes were obtained (Table 7).

Table 7: Mobile phase gradient used with the flash purification system for the prefractionation of the extracts.

Time (min)	MilliQ (%)	Methanol (%)	Acetone (%)	Flash tubes	Fraction number
0-6	95	5	0	1-3	F1
6-12	75	25	0	4-6	F2
12-18	50	50	0	7-9	F3
18-24	25	75	0	10-12	F4
24-36	0	100	0	13-15	F5
36-42	0	50	50	16-18	F6
42-54	0	0	100	19-27	F6

4.4.3 Preparation of SNAP columns for flash fractionation

6.5 g of Diaion® HP-20SS column material (adsorbent) was pre-treated by soaking in 75 mL methanol for minimum 20 minutes before exchanging with MilliQ water, and packing in a flash cartridge using a vacuum manifold. The cartridges were stored at 4 °C until further use.

4.4.4 Stock solution preparation

Materials

Table 8: Equipment used in stock solution preparation.

Equipment	Equipment identification	Supplier
Dimethyl Sulfoxide	D4540	Sigma-Aldrich (St. Louis, MO, USA)
MilliQ water	-	Merck KGaA (Germany)
Heto PowerDry® Freeze Dryer	PL9000	Thermo Fisher Scientific
Büchi Syncore® Polyvap	-	Büchi (Switzerland)
Universal Shaker	SM 30	Edmund Buhler GmbH (Germany)

Method

Subsequently, after pre-fractionation all fractions were dried using Polyvap and stored at -20 °C until further use. The flash fractions were diluted to 40 mg/mL in dimethyl sulfoxide (DMSO) and dissolved using the shaking machine. The DMSO solved fractions were transferred into separate cryo tubes and stored at –20 °C. Deep-well plates (DWPs) were prepared with all the fractions for bioactivity screening. The fractions were first frozen in the DWPs followed by freeze-drying until completely dry. Next, the fractions were dissolved in a 2.5% (v/v) DMSO solution prepared in autoclaved MilliQ water to a concentration of 1 mg/mL, and the plates were stored at -20 °C until further use for bioactivity screening.

4.5 Bioactivity screening of flash fractions

All fractions were screened for antibacterial and anticancer activity. The bioactivity screening of fractions was performed in a class II safety cabinet. The flash fractions were screened for antibacterial activity against the bacterial strains *S. aureus*, *E. coli*, *E. faecalis*, *P. aeruginosa*, *Streptococcus* type B and Methicillin-resistant *S. aureus*, using one concentration (100 µg/mL) in primary screening. The active fractions were re-tested in a secondary secreening using two concentrations (50 and 25 µg/mL) to confirm activity and eliminate the possibility of getting false positives. The flash fractions were screened for anticancer activity against the human melanoma cancer cell line A2058. All fractions were screened in one concentration (100 µg/mL) to investigate activity. The fractions that showed activity were further analysed using preparative RP-HPLC while non-active fractions were excluded from further work. The performance of bioactivity screenings will be described in detail in the subsequent sections.

4.5.1 Antibacterial activity screening

Materials

Table 9: Equipment used for antibacterial activity screening.

Equipment	Equipment identification	Supplier
Brain heart infusion broth (BHI)	53286	Sigma-Aldrich (USA)
Mueller Hinton broth (MH)	275730	Difco
Blood agar plates	-	University hospital of North Norway (UNN)
Luria-Bertoni plates	-	University hospital of North Norway (UNN)
Gentamycin	A 2712	VWR International S.A.S (France)
MilliQ Ultrapure water	-	Merck kGaA (Germany)
Heated Incubator MIR-262	-	Panasonic Healthcare (Japan)
Incubator Unimax	1010	Heidolph Instruments GmbH & Co (Germany)
Victor Multilabel Counter photometric instrument	-	Perkin Elmer, (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (MA, USA)
E. coli	ATCC® 25922	LGC Standards (UK)
S. aureus	ATCC® 25923	LGC Standards (UK)
E. faecalis	ATCC 29212	LGC Standards (UK)
P. aeruginosa	ATCC 27853	LGC Standards (UK)
Streptococcus type B	ATCC® 12386	LGC Standards (UK)
MRSA	ATCC® 43330	LGC Standards (UK)

Method

4.5.1.1 Preparation of the test bacteria

All fractions were screened for antibacterial activity against six human pathogenic bacterial strains (Table 10). The test bacteria were taken out from the freezer, where they were stored at –80 °C, and kept on ice. The bacterial strains were stroked onto blood agar plates and incubated at 37 °C overnight. Afterwards, a scoop of the bacteria from the blood agar plates was transmitted into 8 mL growth medium in falcon tubes and incubated overnight at 37 °C. Different growth medium was used for cultivation of the six bacterial strains (Table 10). 2 mL of the bacterial suspension was transmitted into 25 mL fresh cultivation medium and the cultures were incubated under shaking at 37 °C for the time stated in Table 10 to reach 0.5 McFarland turbidity (1.0×10^8 bacteria/mL). The bacterial solution was diluted 1:1000 in the cultivation medium after incubation.

Table 10: The test bacteria used in the antibacterial activity screening, their cultivation medium, incubation time needed to reach 0.5 McFarland turbidity and CFU ranges.

Bacterial strain	Medium for cultivation	Incubation period (hours)	Bacterial density (CFU ranges)
S. aureus	MH	2.5	0.5-3x10^5 CFU/mL (2500-15000 CFU/well)
E. coli	MH	1.5	0.5-3x10^5 CFU/mL (2500-15000 CFU/well)
E. faecalis	BHI	1.5	0.5-3x10^5 CFU/mL (2500-15000 CFU/well)
P. aeruginosa	MH	2.5	3-7x10^4 CFU/mL (1500-3500 CFU/well)
Streptococcus type B	BHI	1.5	0.5-3x10^5 CFU/mL (2500-15000 CFU/well)
MRSA	BHI	2.5	0.5-3x10^5 CFU/mL (2500-15000 CFU/well)

4.5.1.2 Preparation of the 96-well microtiter plates

Fractions were taken from the test-DWP (see section 4.4.4 ‘’Stock solution preparation’’) and diluted with autoclaved MilliQ water to the selected concentrations. All fractions were screened at a concentration of 100 µg/mL (in duplicates) in the primary screening. The active fractions were re-tested in a secondary screening at 50 and 25 µg/mL (in duplicates). 50 µL of the fractions were transferred to 96-well microtiter plates, one plate was used for each bacterial strain that was tested. Subsequently, 50 µL of the prepared 1:1000 bacterial solutions were added to the fractions. In addition, positive and negative controls for the screening were included on the plates. Negative control (N) was added to the first column on the plate and it was prepared with 50 µL growth medium and 50 µL autoclaved MilliQ, whereas positive control (P) was added to the last column on the plate and it was prepared with 50 µL bacterial suspension and 50 µL autoclaved MilliQ. The microtiter plates were incubated for 20-24 hours at 37 °C.

Table 11: Setup for MIC assay. N is negative control. P is positive control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	1	1	9	9	17	17	25	25	33	33	P
B	N	2	2	10	10	18	18	26	26	34	34	P
C	N	3	3	11	11	19	19	27	27	35	35	P
D	N	4	4	12	12	20	20	28	28	36	36	P
E	N	5	5	13	13	21	21	29	29	37	37	P
F	N	6	6	14	14	22	22	30	30	38	38	P
G	N	7	7	15	15	23	23	31	31	39	39	P
H	N	8	8	16	16	24	24	32	32	40	40	P

4.5.1.3 Gentamycin control

For controlling and validating the stability of the bacterial strain a gentamycin control of the setup was used. The control was performed in the following concentrations: 16 µg/mL, 10 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.13 µg/mL, 0.06 µg/mL, 0.03 µg/mL and 0.02 µg/mL, to determine the minimum inhibitory concentration of gentamycin for the test bacteria. 50 µL gentamycin control was transferred to 50 µL bacterial solution in a 96-well microtiter plate and the plate was incubated at 37 °C overnight. The MIC values were determined by visual inspection of growth inhibition. The screening had to be run again if the MIC values (Table 12) for gentamycin were beyond the reference values by more than one titer step.

Table 12: Reference MIC values for the test bacteria used in the antibacterial screening.

Test bacteria	Reference MIC values for gentamycin (µg/mL)
S. aureus	0.06
E. coli	0.13
E. faecalis	8
P. aeruginosa	0.25
Streptococcus type B	4
MRSA	0.015

4.5.1.4 Control of colony forming unit

After incubation of 1.5 hours, the bacterial solution was diluted 100 000 and 1 000 000 times in 0.9% sodium chloride solution. Furthermore, 100 µL of the diluted suspensions were stroked out in two parallels on Luria-Bertoni plates and incubated at 37 °C overnight. Afterwards, the quantity of colonies was counted, and the bacterial density (CFU) was determined and

compared to the standard CFU ranges (Table 10). The bacterial growth was deemed normal if the calculated CFU were within the range of the standard CFU ranges.

4.5.1.5 Reading of plates and evaluation of results

The microtiter plates were observed visually for growth inhibition after incubation for 20-24 hours. Subsequently, the photometric instrument was used to measure the absorbance of the microtiter plates at 600 nm (Abs_{600}). The software WorkOut 2.5 was utilised to process the results. The fractions were characterised either as active, questionable or inactive based on the Abs_{600} values.

- Active < 0.05
- Questionable 0.05-0.09
- Inactive > 0.09

Fractions considered active or questionable were re-tested in the secondary screening.

4.5.2 Anticancer activity screening

Materials

Table 13: Equipment used in the anticancer activity screening.

Equipment	Equipment identification	Supplier
Roswell Park Meomorial Institue medium (RPMI-1640)	FG 1383	Merck KGaA (Germany)
Fetal Bovine Serum (FBS)	S0115	Merck KGaA (Germany)
A2058 Melanoma	ATCC® CRL-11147™	LGC Standards (UK)
CellTiter 96® Aqueous One Solution Reagent (AQOS)	G358B	Promega (Wisconsin, USA)
Gentamycin (10 mg/mL)	A 2712	Merck KGaA (Germany)
L-Alanyl-L-Glutamine (200 nM)	K 0302	Merck KGaA, Germany
Triton™ X-100	T8787	Sigma-Aldrich (Missouri, USA)
Dulbecco's Modified Eagle Medium (D-MEM)	32430027	Thermo Fisher Scientific (Massachusetts, USA)
Sanyo CO_2 Incubator MCO-18AIC	-	Panasonic Biomedical (Japan)
DTX Multimode Detector	880	Beckman Coulter (California, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)

Method

The cells were seeded in a 96-well microtiter plate in D-MEM medium with 10% FBS, gentamycin (10 µg/mL) and 1% L-Alanyl-L-glutamine, at a concentration of 2000 cells/wells. The plates were incubated at 37 °C with 5% CO_2 for 24 hours. After incubation, the medium was replaced with RPMI-1640 medium with 10% FBS, gentamycin (10 µg/mL) and 1% L-Alanyl-L-glutamine. Fractions were taken from the test-DWP (see section 4.4.4 ''Stock Solution Preparation'') and diluted in RPMI medium to the chosen concentration. 100 µL of the fractions were added to the cells in triplicates at a concentration of 100 µg/mL (Table 14). Negative controls (N) made with RPMI-1640 medium containing 10% FBS and positive controls (P) made with 1% triton were included in the plates as well. The plates were further incubated for 72 hours at 37 °C with 5% CO_2. Afterwards, 10 µL Aqueous One Solution was added to each well, and the plates were incubated for 1 additional hour.

Table 14: Setup for cancer assay. Two sets of 8 fractions in triplicates were seeded in each plate. "a" indicates the first set of fractions. "b" indicates the second set of fractions. (N) indicates negative control. (P) indicates positive control. The grey areas indicate wells containing only medium.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1_a	2_a	3_a	4_a	5_a	6_a	7_a	8_a	(N)		
C		1_a	2_a	3_a	4_a	5_a	6_a	7_a	8_a	(N)		
D		1_a	2_a	3_a	4_a	5_a	6_a	7_a	8_a	(N)		
E		1_b	2_b	3_b	4_b	5_b	6_b	7_b	8_b	(N)		
F		1_b	2_b	3_b	4_b	5_b	6_b	7_b	8_b			(P)
G		1_b	2_b	3_b	4_b	5_b	6_b	7_b	8_b			(P)
H												(P)

4.5.2.1 Reading of plates and evaluation of results

The absorbance was recorded at 485 nm using the DTX 880 Multimode Detector, and cell survival calculated using equation 1. The fractions were characterised either as active, questionable or inactive based on the cell survival.

$$\%\ cell\ survival = \frac{(At - Ab)x100}{(Ac - Ab)}$$

Equation 1: Calculation of cell survival (%). At is the average absorbance value measured in wells with fractions, Ab is the average absorbance value measured in wells with positive control, and Ac is the average absorbance measured in wells with negative control.

- Active < 50% cell survival
- Questionable 50-60% cell survival
- Inactive > 60% cell survival

4.6 Re-fractionation of active flash fractions

Materials

Table 15: Equipment used in re-fractionation of the flash samples.

Equipment	Equipment identification	Supplier
Acetonitrile	75-05-8	Merck KGaA (Germany)
MilliQ	-	Merck KGaA (Germany)
Formic acid	64-18-6	Sigma-Aldrich (St. Louis, MO, USA)
Methanol	20864	VWR International S.A.S (France)
Preparative HPLC-MS system	-	Waters (MA, USA)
HPLC pump	515	Waters (MA, USA)
Controller	600	Waters (MA, USA)
Mass Detector	3100	Waters (MA, USA)
Photo Diode Array Detector	2996	Waters (MA, USA)
Sample Manager (Fraction controller)	2767	Waters (MA, USA)
Xterra® Shield RP 18 OBD™ prep column	-	Waters (MA, USA)
Software: MassLynx	Version 4.1	Waters (MA, USA)
Express SpeedVac Concentrator	SC250	Thermo Fisher Scientific (MA, USA)
Branson Ultrasonic Cleaner	3510-DTH	Emerson Industrial Automation (Missouri, USA)

Method

The re-fractionation of the active flash fractions was done on a preparative HPLC-MS system. The system was composed of a HPLC-pump, an in-line-solvent degasser, a fraction-collector and injector, a UV/Vis-flow-spectrometer and a mass detector. The HPLC-MS instrument was tuned and optimised using the software MassLynx version 4.1. The source temperature was 120 °C, the desolvation temperature was 300 °C and the desolvation gas flow rate was set to 650 L/hr. The capillary and cone voltages were 15 V and 40 V, respectively. HPLC separations were performed on a 125 Å, 10 µm, 10 mm × 300 mm X-Terra Shield C18 column at a flow rate of 6 mL/min. Re-fractionation was arranged to collect one fraction per minute, with a total of 40 fractions collected per analysis and 6 mL in each fraction, with a gradient of two mobile phases:

- Mobile phase A: MilliQ H_2O with 0.1% (v/v) formic acid
- Mobile phase B: Acetonitrile with 0.1% (v/v) formic acid

Different mobile phase gradients were used for the flash fractions to obtain efficient separation of the compounds in each of them. The gradient used for the flash fractions labelled 'X0845_05' and 'X0811B_05' was 25% B to 100% B in 32 minutes, while the gradient used for the flash fraction labelled 'X0834B_05' was 15% B to 100% B in 32 minutes. Before injection, the

fractions were diluted with 80% methanol to reduce their viscosity. 880 µL of the flash fraction labelled 'X0845_05' was injected, while 750 µL of the flash fractions labelled 'X0811B_05' and 'X0834B_05' were injected. The injection volume was selected by a ''trial-and-error'' approach. The fractions obtained will be referred to as subfractions further in the thesis.

4.6.1 Treatment of subfractions after re-fractionation

After re-fractionation, each subfraction was split between three DWPs for bioactivity screening. Each fraction contained 6 mL, so 2 mL of each fraction was transferred to each DWP using an electric 1-10 mL pipette with the stepper settings 3*2 mL. Next, the DWPs were vacuum centrifuged in the SpeedVac for 3 hours to remove organic solvents. The plates were placed in the freezer at -80 °C overnight, before they were freeze-dried for 24 hours to remove the remaining water. The subfractions were dissolved in 300 µL 1% (v/v) DMSO aq., and the plates were stored at -20 °C until further use for bioactivity screening.

4.7 Bioactivity screening of subfractions

Antibacterial and anticancer activity screening were performed as described in section 4.5.1 ''Antibacterial activity screening'' and section 4.5.2 ''Anticancer activity screening'', respectively. However, the subfractions were only screened for antibacterial activity against the bacterial strains *E. coli* and *Streptococcus* type B. In addition, only fractions from the bacterial strains M09W202 and M10B807 were screened for anticancer activity.

4.8 Dereplication of active subfractions from bioactivity screening

Materials

Table 16: Equipment used in the dereplication process.

Equipment	Equipment identification	Supplier
HPLC glass vials	-	Waters (Milford, MA, USA)
VION® IMS QToF	-	Waters (Milford, MA, USA)
Acquity UPLC Column Manager	-	Waters (Milford, MA, USA)
Acquity UPLC PDA Detector	-	Waters (Milford, MA, USA)
Acquity UPLC BEH C18 Column	-	Waters (Milford, MA, USA)
Software: UNIFI	Version 1.8.2	Waters (Milford, MA, USA)
MilliQ water	-	Merck KGaA (Germany)
Formic acid	64-18-6	Sigma-Aldrich (St. Louis, MO, USA)
Acetonitrile	75-05-8	Merck KGaA (Germany)
Methanol	20864	VWR International S.A.S (France)

Method

The subfractions considered active in the bioactivity screening were analysed using UHPLC-QToF-MS. 50 µl of the subfractions were transferred to HPLC glass vials and injected in the UHPLC system. The flow rate was maintained at 0.450 mL min^{-1} and a volume of 5 µL of each fraction was injected. Separation was obtained on a C18 column (2.1 × 100 mm, 1.7 µm) with a linear gradient consisting of MilliQ water (A) and acetonitrile (B), both containing 0.1% (v/v) formic acid, starting at 10% B and increased to 100% B in 12 minutes. The samples were run in both, positive and negative ion mode (ESI^- and ESI^+), in separate runs. N_2 was used as the desolvation gas. The desolvation temperature was 450 °C, the flow rate was 800 L/h and the source temperature was 100 °C. The capillary and cone voltages were 0.80 kV and 40 V, respectively. The data were collected for each test sample across a mass range of 50-2000 *m/z* with a scan time of 0.2 seconds.

The active subfractions were compared to the inactive ones, in order to identify compounds found particularly, or in a significantly higher concentration in the active subfractions. This was done in an attempt to determine possible compounds responsible for the observed activity. The software Waters UNIFI version 1.8.2 was utilised to calculate a possible elemental composition for these compounds, based on accurate masses and the isotopic pattern of the compounds. Furthermore, the calculated elemental compositions were used to search databases *via* ChemSpider, mainly Marine Drugs, Marinlit, Natural Product Updates, Nature Chemical Biology and Nature Chemistry, to assess whether the compounds were new or previously discovered.

4.9 Upscaling and purification of possible bioactive compounds

Materials

Table 17: Equipment used in the purification of possible active compounds.

Equipment	Equipment identification	Supplier
Acetonitrile	75-05-8	Merck KGaA (Germany)
MilliQ	-	Merck KGaA (Germany)
Formic acid	64-18-6	Sigma-Aldrich (St. Louis, MO, USA)
Methanol	20864	VWR International S.A.S (France)
preparative HPLC-MS system	-	Waters (MA, USA)
HPLC pump	515	Waters (MA, USA)
Controller	600	Waters (MA, USA)
Mass Detector	3100	Waters (MA, USA)
Photo Diode Array Detector	2996	Waters (MA, USA)
Sample Manager (Fraction controller)	2767	Waters (MA, USA)
X-Terra® Shield RP 18 OBD™ prep column	-	Waters (MA, USA)
XSelect™ CSH™ Fluoro-Phenyl prep column	-	Waters (MA, USA)
SunFire™ Shield RP 18 OBD™ prep column	-	Waters (MA, USA)
SpeedVac Concentrator	SC250	Thermo Fischer Scientific (MA, USA)

Method

The purification of the compounds of interest was carried out by utilising the same HPLC-MS equipment, software and mobile phases as described in section 4.6 ‘’Re-fractionation of active flash fractions’’. The purification was run in two rounds in order to obtain material of sufficient purity for further investigation. Various columns were used at a flow rate of 6 mL/min in an attempt to isolate and purify these compounds (Table 18). The injection volume was selected by a ‘’trial-and-error’’ approach, varying between 100-400 µL for the isolated compounds. The mobile phase gradients used for purification of the compounds are listed in Table 18, and they were selected based on results from previous runs.

The isolated compounds were collected in fractions using time or mass triggered fractionation and dried by vacuum centrifugation in the SpeedVac. They were resuspended in 400 µL methanol and 100 µL DMSO for the second purification round. In the second purification round, the compounds were collected in fractions using mass triggered fractionation. After purification, the compounds were dried in the SpeedVac and dissolved in 1% (v/v) DMSO aq. for bioactivity screening.

Table 18: Column, gradient and run-time used for the first and the second isolation rounds of the different compounds.

Compound	Separation round	Column	Gradient (%) ACN	Time (min)
TS-7-mix	First	X-Terra	15-100	32.00
TS-7-317	First	X-Terra/SunFire	15-100/20-100	32.00/32.00
TS-7-281	First	X-Terra/SunFire	15-100/20-100	32.00/32.00
	Second	Fluoro-phenyl	20-100	15.00
TS-7-558	First	Fluoro-phenyl	20-100	15.00
TS-7-rt10-13	First	Fluoro-phenyl	20-100	15.00
TS-19-227	First	X-Terra/SunFire	15-100/20-100	32.00/32.00
	Second	Fluoro-phenyl	20-100	15.00
TS-25-398	First	X-Terra	15-100	32.00
	Second	Fluoro-phenyl	50-100	15.00
TS-25-386	First	X-Terra	15-100	32.00
	Second	Fluoro-phenyl	50-100	15.00
TS-4-652	First	X-Terra	50-100	15.00
	Second	Fluoro-phenyl	10-40	15.00
TS-4-265	First	Fluoro-phenyl	10-40	15.00
AM-23-404	First	SunFire	50-100	15.00
	Second	Fluoro-phenyl	50-100	15.00
AM-24-404	First	SunFire	50-100	15.00
	Second	Fluoro-phenyl	50-100	15.00
AM-25-418	First	SunFire	50-100	15.00
	Second	Fluoro-phenyl	50-100	15.00

/, indicates that the compounds were isolated in two rounds using different columns and elution gradients. The compounds are named based on the bacterial strain they are isolated from, the subfractions in which they were eluting and their *m/z* values or retention time. TS indicates that the compounds are isolated from *Thalassospira*. AM indicates that the compounds are isolated from *Alteromonas*.

4.10 Bioactivity screening of the isolated compounds

The isolated compounds considered responsible for the observed activity were screened for antibacterial activity as described previously in section 4.5.1 ‘’Antibacterial activity screening’’ in order to confirm their activity. The compounds were screened for antibacterial activity against the bacterial strain *Streptococcus* type B in three different concentrations (50, 10 and 5 µg/mL). However, the compounds TS-7-281, TS-7-317, TS-7-558, and TS-7-rt10-13 were only tested in 50 µg/mL, and they were also screened for anticancer activity against the cell line A2058 as described in section 4.5.2 ‘’Anticancer activity screening’’ in 50 µg/mL. Moreover, the compounds AM-23-404, AM-24-404 and AM-25-418 were submitted to NMR analaysis before performing bioassays to confirm their activities.

4. 11 Structure elucidation of target compounds by NMR

This part of the project was performed by Johan Isaksson at the Department of Chemistry at UiT The Arctic University of Norway.

All NMR spectra were acquired on a Bruker Avance III HD spectrometer equipped with an inverse detected TCI probe with cryogenic enhancement on 1H, 2H and 13C, operating at 599.90 MHz and 150.86 MHz for 1H and 13C, respectively. Samples were prepared in DMSO-d6 and methanol-d4, and recorded at 298 K. All experiments were acquired using standard pulse sequences for Proton, Presat, Carbon, DQFCOSY, ECOSY, HSQC (bip), HMBC (bip), H2BC (bip), HSQCTOCSY (mlev), TOCSY (clean mlev), NOESY and ROESY (adiabatic) in Topspin 3.5pl7, using gradient selection where applicable, and processed in Mnova 12.0.0. Spectra were referenced on the residual solvent peak of methanol-d4 (δH = 3.31 and δC = 49.00) or DMSO-d6 (δH = 2.50 and δC = 39.52).

5.0 Results

5.1 Extraction of the bacterial cultures

The secreted secondary metabolites produced by the bacteria were harvested from the culture media using Diaion® HP-20 resin and extracted with methanol, and the extracts were dried under reduced pressure for further investigation. The weight of the crude extracts obtained from the bacterial strains AM, PM and TS were 6.05 g, 18.33 g and 17.75 g, respectively.

5.2 Pre-fractionation of the extracts

The crude extracts from the bacterial strains were pre-fractionated into six flash fractions, where only flash fraction 5 was used further in the thesis as the other fractions did not display any activity. The weight of flash fraction 5 obtained from the bacterial strains AM, PM and TS were 34 mg, 4081.7 mg and 380.8 mg, respectively.

5.3 Bioactivity screening of flash fractions

Antibacterial activity was first tested in one concentration (100 µg/mL). Fraction 5 was found to be active, and it was therefore re-tested at final concentrations of 50 µg/mL and 25 µg/mL. Fraction 5 from the extracts of PM and AM showed inhibitory activity against the bacterial strains *E. faecalis* and *Streptococcus* type B (Fig. 12, 13 and 14). However, fraction 5 of PM had only slight activity (questionable) against the bacterial strain *E. faecalis* and no activity was observed against this strain at the lowest tested concentration (Fig. 14). Fraction 5 from the extract of AM also showed activity against MRSA at the highest tested concentrations (Fig. 12 and 13). As for fraction 5 from the extract of TS, slight activity (questionable) against the bacterial strain MRSA was observed at concentrations of 100 and 50 µg/mL (Fig. 12 and 13). Questionable fractions were treated as active and were further analysed. None of the fractions displayed activity against Gram negative test strains (results not shown). Furthermore, only fraction 5 of the extracts of PM and AM displayed anticancer activity (Fig. 15). Hence, fraction 5 from the extracts of all three bacterial strains were further investigated for antibacterial activity, whereas only fraction 5 of the extracts of the bacterial strains PM and AM were further investigated for anticancer activity. No antibiotic or anticancer activity was observed for any of the other fractions as can be seen in Fig. 12, and they will thus not be mentioned any further.

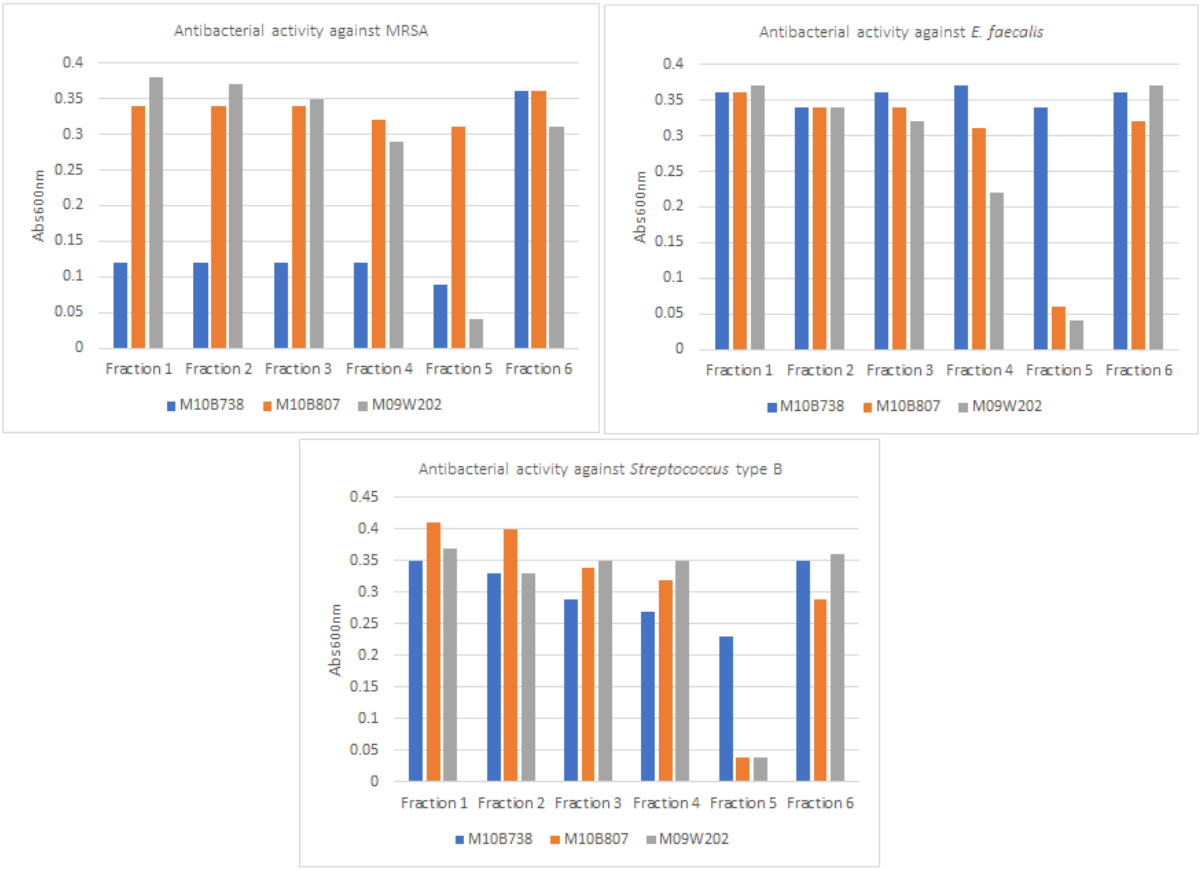

Figure 12: The antibacterial activity of flash Fractions 1-6 from the extracts of AM (M09W202), PM (M10B807) and TS (M10B738) were tested in a growth inhibition assay. Results from the screening against gram negative strains are not shown. The test concentration was 100 µg/mL. Antibacterial assay: Active, $Abs_{600\ nm} < 0.05$; Questionable, $Abs_{600\ nm}$ 0.05-0.09; Inactive, $Abs_{600\ nm} > 0.09$.

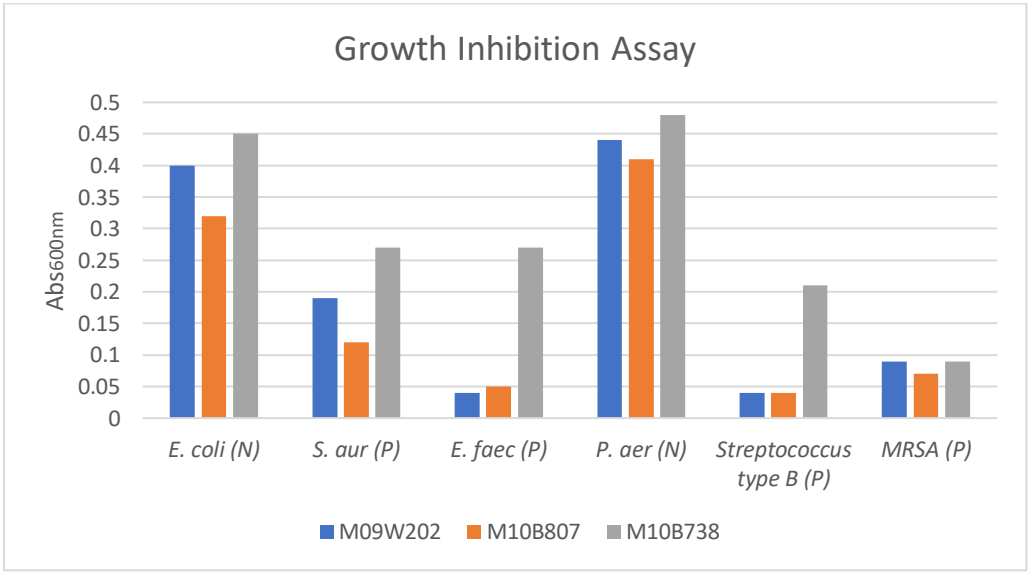

Figure 13: The antibacterial activity of flash Fraction 5 from the extracts of AM (M09W202), PM (M10B807) and TS (M10B738) was tested in a growth inhibition assay. The test concentration was 50 µg/mL. Antibacterial assay: Active, $Abs_{600\ nm} < 0.05$; Questionable, $Abs_{600\ nm}$ 0.05-0.09; Inactive, $Abs_{600\ nm} > 0.09$. (P) Gram positive, (N) Gram negative.

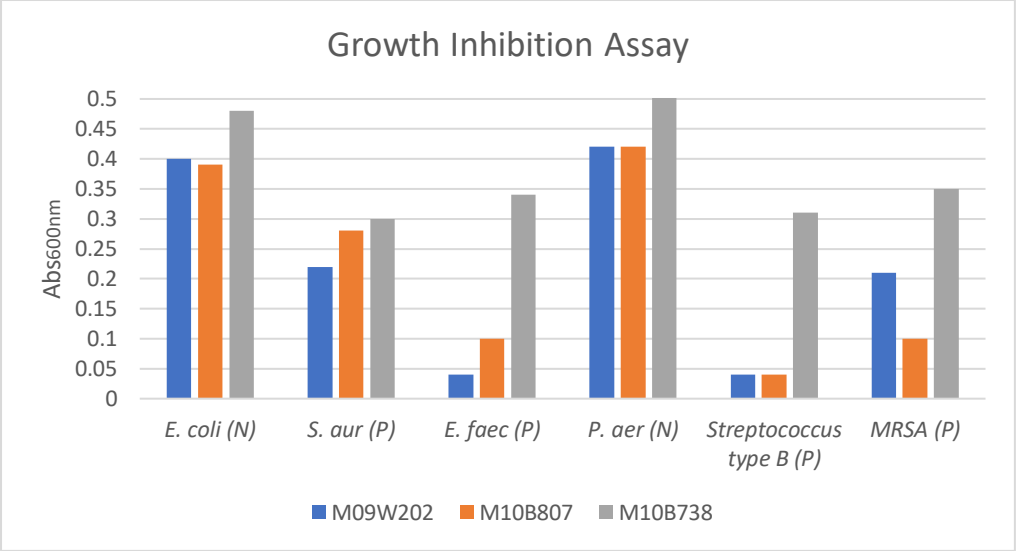

Figure 14: The antibacterial activity of flash Fraction 5 from the extracts of AM (M09W202), PM (M10B807) and TS (M10B738) was tested in a growth inhibition assay. The test concentration was 25 µg/mL. Antibacterial assay: Active, $Abs_{600\ nm}$ < 0.05; Questionable, $Abs_{600\ nm}$ 0.05-0.09; Inactive, $Abs_{600\ nm}$ > 0.09. (P) Gram positive, (N) Gram negative.

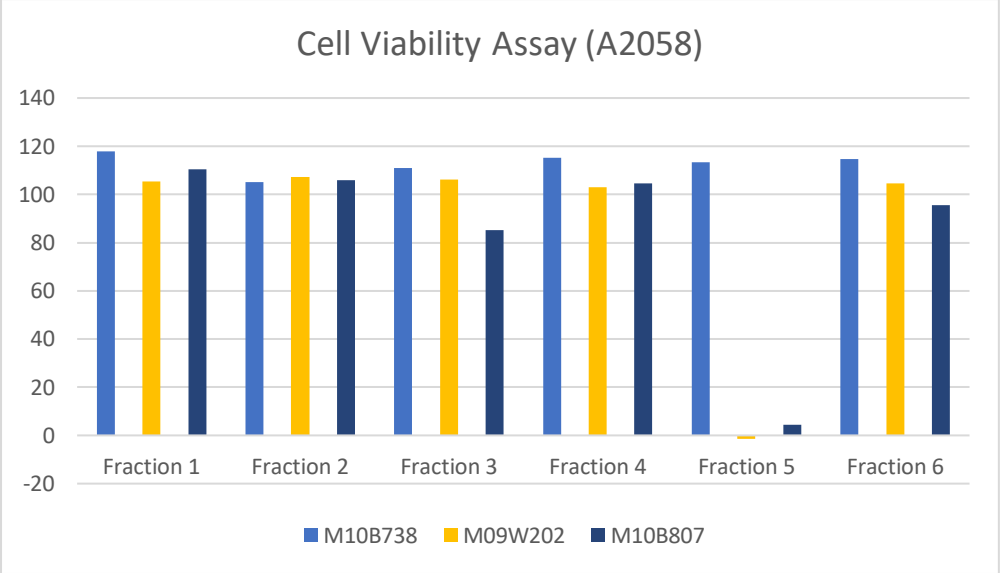

Figure 15: The anticancer activity of flash Fractions 1-6 from the extracts of AM (M09W202), PM (M10B807) and TS (M10B738). Anticancer activity of the fractions were estimated with a cell viability assay. The tested concentration was 100 µg/mL. Viability assay: Active, < 50% cell survival; Inactive, > 50% cell survival.

5.4 Re-fractionation of active flash fractions

It was not possible to assign the bioactivity observed in flash fractions with sufficient certainty to any known compound in the dereplication process. Thus, the active flash fractions were further fractionated (recording an ESI+/- mass chromatogram) into 40 subfractions in an

attempt to identify the compounds responsible for activity *via* bioassay guided purification. The resulting BPI chromatograms can be seen in Fig. 16.

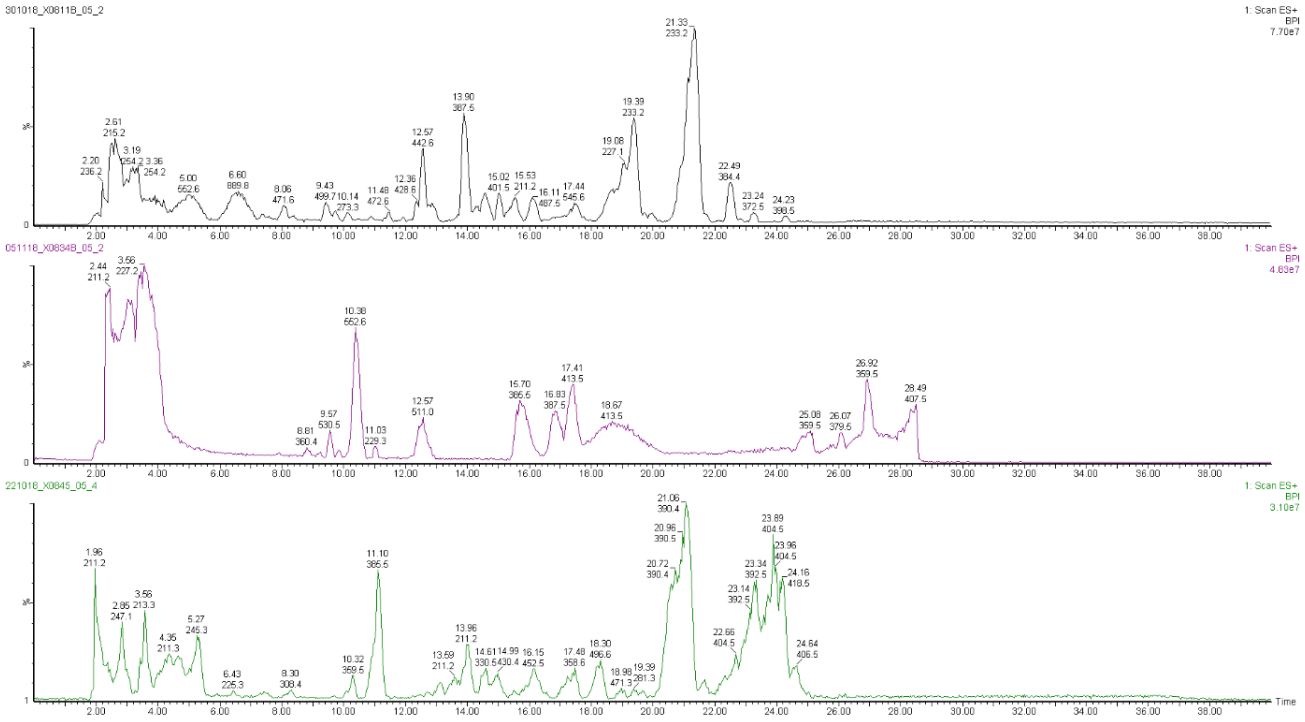

Figure 16: BPI chromatograms (ESI⁺) from the re-fractionation of flash fraction 5 from the bacterial strains TS (top), PM (middle) and AM (bottom), with prep HPLC-MS. The fractions were injected onto a Waters X-Terra® Prep RP18 (10 µm 10x300 mm) column. A mobile phase gradient of MilliQ water and Acetonitrile, both with 0.1% (v/v) FA, was used with a flow rate of 6 mL/min. 40 subfractions were collected from each flash fraction.

5.5 Bioactivity screening of subfractions

The subfractions were screened for antibacterial and anticancer activity in order to select which fractions to focus on and which to terminate.

5.5.1 Antibacterial activity screening of subfractions

The 40 subfractions obtained by preparative HPLC were tested for antibacterial activity against the two bacterial strains *E. coli* and *Streptococcus* type B. The subfractions were first tested diluted in 300 µL 1% (v/v) DMSO aq., showing a high number of active fractions occuring in a sequence, particularly against the bacterial strain *Streptococcus* type B (results not shown). The high activity was suspected to be caused by organic compounds not of interest or it could be that the same active compounds were spread over several subfractions. Hence, the subfractions in each DWP were tested diluted 1:10 in 1% (v/v) DMSO to investigate if the results were reproducible, and as high dilution may result in detection of

potent active compounds. Therefore, we took out 100 µL of the dissolved fractions and diluted it with 900 µL 1% (v/v) DMSO in water to a 1:10 dilution in a new DWP for re-testing. The 1:10 dilution has resulted in much less activity. The subfractions 23-27 from the extract of AM were active against the bacterial strain *Streptococcus* type B. The subfractions 19-32 from the extract of PM showed activity against the same test strain. The subfractions 3-11 and 25-27 from the extract of TS displayed activity against the same test strain as well. None of the fractions displayed activity against *E. coli* (results not shown).

5.5.2 Anticancer activity screening of subfractions

The subfractions from extracts of PM and AM were screened for anticancer activity diluted first in 300 µL 1% (v/v) DMSO, resulting in observed activity in the subfractions 24-31 from the extract of PM, and no activity for any of the subfractions from the extract of AM. The subfractions were further diluted 1:10 in 1% (v/v) DMSO as described under 5.5.1 ''Antibacterial activity screening of subfractions'' and screened for anticancer activity again, resulting in no activity for any of the subfractions neither from PM or AM (results not shown).

5.6 Dereplication of active subfractions from bioactivity screening

Several compounds detected in the active subfractions could be responsible for the observed activity. However, some of the compounds were highlighted as most likely active as they were exclusively present, or present in a considerably higher concentration in active fractions compared to inactive fractions and were thus, selected for further investigation. To identify the compounds that possibly caused the observed antibiotic activity in the bioassay, the active subfractions in which these compounds were eluting, were analysed by HR-MS. Potentially active compounds were isolated and tested for bioactivity to be certain whether they were responsible for the observed activity or not. The MS chromatogram of some fractions considered active, showed only background noise and no signals were observed neither in positive or negative ESI mode, such as the fractions AM-26 and 27, PM-30 and TS-8 to 11. Compounds were however detected in the UV/Vis detector. These were excluded from further study as they were suspected of being lipids, fatty acids and other compounds not of interest, according to the experience of Marbio. The subfractions selected for dereplication with HR-

MS based on the results from antibacterial activity screening and the comparison of active fractions with inactive ones were:

- TS-3, TS-4, TS-7, TS-25, TS-26 and TS-27
- AM-23, AM-24 and AM-25
- PM-19, PM-25, PM-27 and PM-29

5.6.1 Compounds selected for isolation from the bacterial strain TS

Subfraction 3

No compounds of interest were found in this subfraction as metabolites with unspecific effects were suspected to be responsible for the observed bioactivity and as a result, the subfraction was excluded from further work. Moreover, several of the compounds present in this fraction were found to be growth media components.

Subfraction 4

The mass signal *m/z* 652.6 ($[M+H]^+$) detected in the active subfraction 4 from the bacterial strain TS was believed to be responsible for the observed activity in this fraction, as it was particularly present in the fraction in relatively high concentration. The calculated elemental composition for this compound was $C_{26}H_{73}N_{19}$, which gave several hits in database searches. Together with another compound with *m/z* 265.2 it was identified as a growth media component and both were excluded from further work.

Subfraction 7

Several compounds were candidates for the observed activity in subfraction TS-7. Thus, all the compounds of interest were isolated in a fraction named TS-7-mix for bioactivity screening in order to investigative whether some of them were active. The compounds most likely responsible for the observed activity were isolated and re-tested for activity as pure compounds for bioactivity confirmation. This fraction was compared to that of the growth medium used in cultivation of the bacterial strain TS in order to avoid selecting growth media components for isolation. Two compounds with *m/z* 281.6 and 317.4 were suspected of being responsible for the observed bioactivity based on the results from dereplication and comparison of this fraction with that of the growth medium. No elemental composition was calculated for these compounds in HR-MS, so they were selected for isolation and characterisation by NMR analysis, since they were not identified in dereplication.

Subfraction 19

In flash fraction 5 from the extract of TS, a compound with *m/z* 227.01 ($[M+H]^+$) (Fig. 17) of interest was detected, and despite that the subfraction TS-19 in which it was eluting did not display any antibacterial activity, it was decided to isolate this compound for structure elucidation due to interesting chemical properties, as well as it potentially being active in other assays. The mass spectrum of this compound can be seen in Fig. 17-right, showing a pattern with three signals, *m/z* 227, 229 and 231, with a 9:6:1 ratio and separated by two mass units. This isotope pattern indicates that the compound contains two Cl atoms in its structure. The elemental composition was calculated to be $C_{10}H_8N_2Cl_2$, and it was suggested to be an aminopyrrolnitrin in databases, several other hits were however given indicating uncertainty about its identity. The compound was therefore isolated for further investigation.

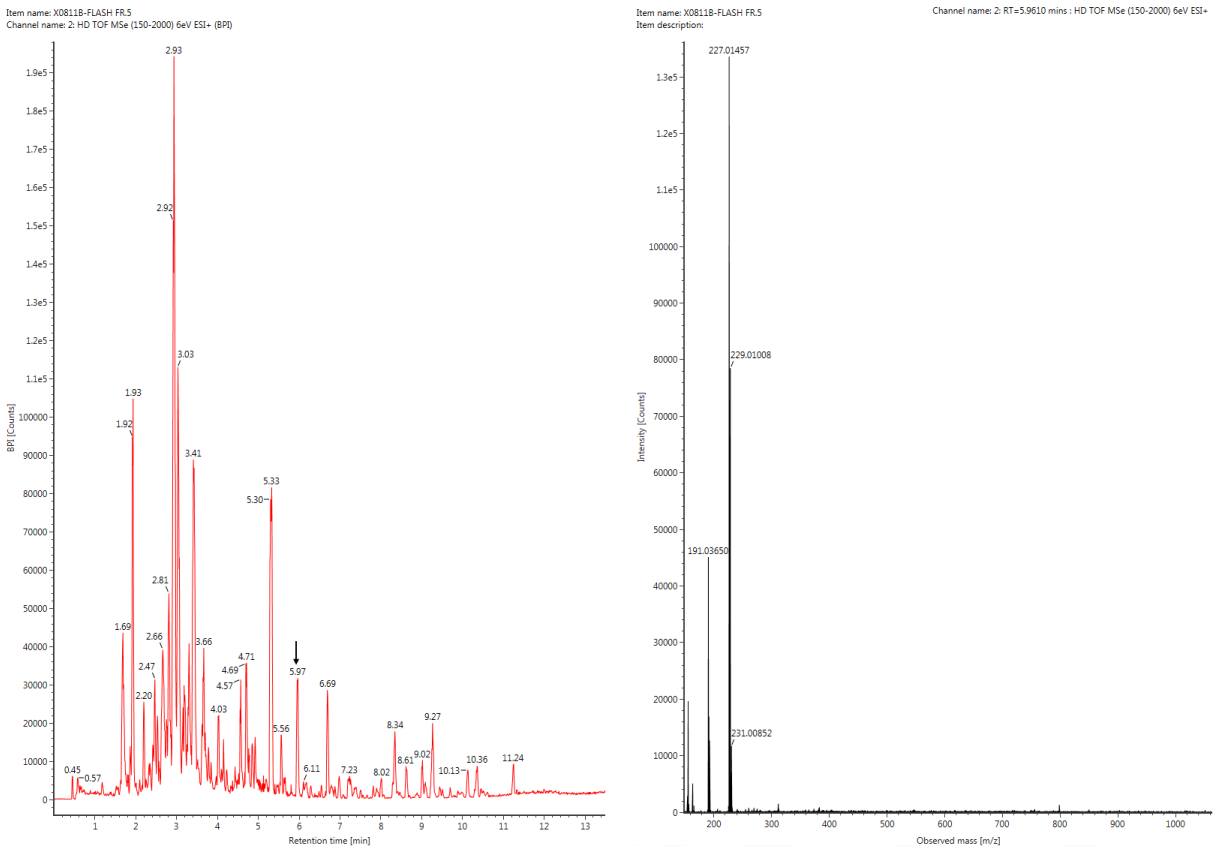

Figure 17: Left) HR-MS BPI chromatogram of the active flash fraction 5, in positive ESI mode. The fraction was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient of MilliQ water with 0.1% (v/v) FA and Acetonitrile with 0.1% (v/v) FA was used with a flow rate of 0.45 ml/min. Right) Mass spectrum for the compound TS-19-227 (marked with a black arrow in the chromatogram).

Subfraction 25

Two signals with *m/z* 398.3 ($[M+H]^+$) and 386.3 ($[M+H]^+$) were detected in the active subfraction 25 from the extract of TS (Fig. 18). These compounds were suspected to possibly be responsible for the observed activity in this fraction, as they were exclusively present in the fraction in high concentrations and were not observed in any of the inactive fractions. The mass spectra of these compounds are given in Fig. 18-right. Their calculated elemental compositions were $C_{22}H_{39}NO_5$ and $C_{21}H_{39}NO_5$, respectively. These formulas gave no hits in the most commonly used databases. Hence, these compounds were submitted to isolation and purification using preparative HPLC-MS for further investigation, and they were named TS-25-398 and TS-25-386, respectively.

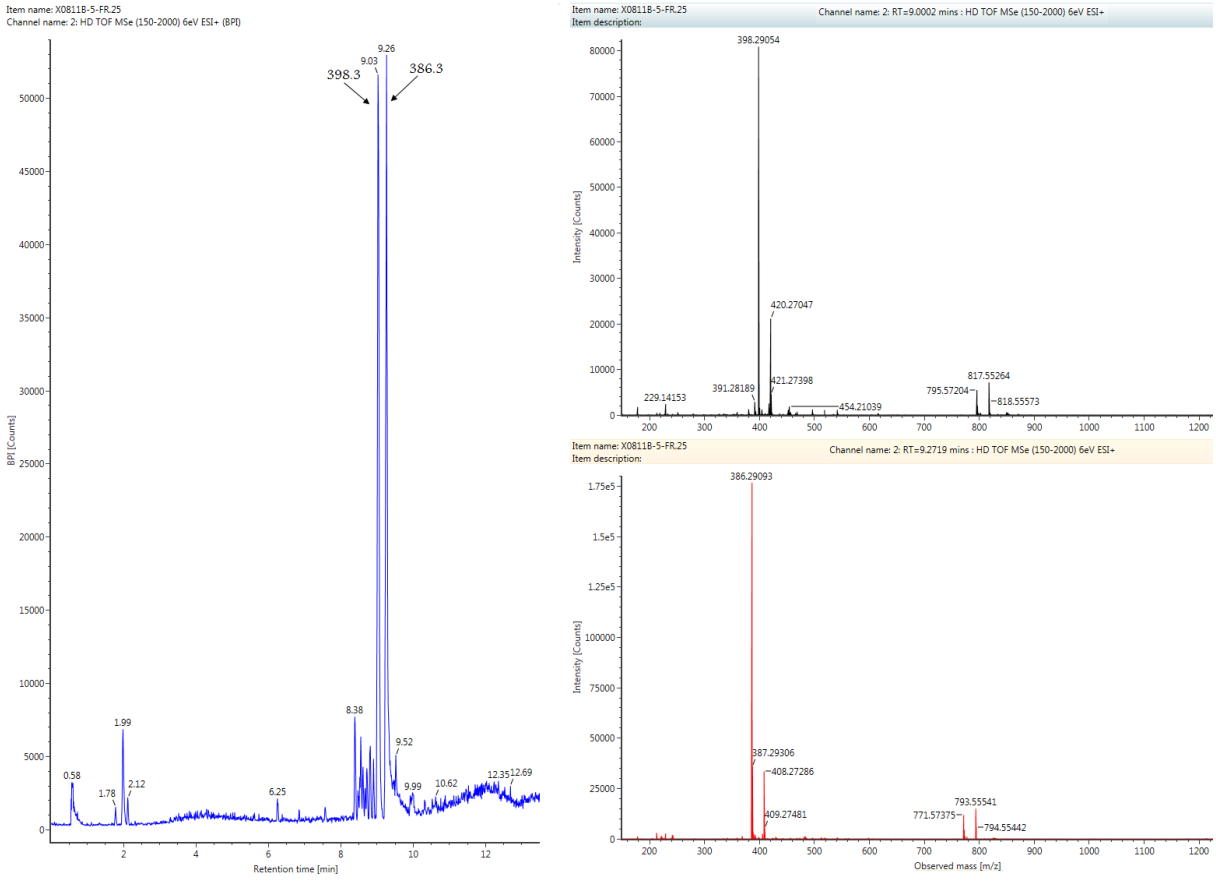

Figure 18: Left) HR-MS BPI chromatogram of the active fraction TS-25, in positive ESI mode. The fraction was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient of MilliQ water with 0.1% (v/v) FA and Acetonitrile with 0.1% (v/v) FA was used with a flow rate of 0.45 ml/min. Right) Mass spectrum for the compounds 398.3 m/z and 384.3 m/z. The peaks with m/z 420.3 and 408.3 are sodium adducts.

Subfractions 26-27

No compounds of interest were found in these subfractions as lipids were suspected to be responsible for the observed bioactivity, and as a result the subfractions were excluded from further work. Moreover, several of the compounds present in these fractions were found to be growth media components.

5.6.2 Compounds selected for isolation from the bacterial strain PM

In the active subfractions PM-19 and PM-25, two peaks with *m/z* 553.3369 ($[M+Na]^+$) and 555.3502 ($[M+Na]^+$) were detected. Their calculated elemental compositions were, $C_{28}H_{50}O_9$ and $C_{28}H_{52}O_9$, respectively, giving several hits in database searches. Their retention times, masses and elemental compositions were consistent with Rhamnolipids (RLs), which have been previously isolated from a different bacterial strain at Marbio, including a *Pseudomonas* species (94). RLs have been reported to exhibit various bioactivities including antimicrobial and anticancer, they were thus suggested to be responsible for the observed activity. Further work on these fractions was as a consequence of this terminated. Moreover, a peak with *m/z* 381.2611 ($[M+Na]^+$) was detected in the active subfractions PM-27 and PM-29. The calculated elemental composition of this compound was $C_{20}H_{38}O_5$, and it was identified as a RL based on previous findings. This compound was hence believed to be responsible for the observed activity. As a result, no compounds were isolated from this bacterial strain.

5.6.3 Compounds selected for isolation from the bacterial strain AM

In the active subfractions AM-23 and AM-24, two peaks with *m/z* 555.3499 ($[M+Na]^+$) and 555.3508 ($[M+Na]^+$) were detected (marked with black arrows in Fig. 19). The calculated elemental composition of both compounds was $C_{28}H_{52}O_9$, indicating RLs, which is consistent with previous findings. Another RL with *m/z* 555.3494 ($[M+Na]^+$) was detected in the active subfraction AM-25 (marked with a black arrow in Fig. 19) and its elemental composition was calculated to be the same as the two other RLs. The RL was however present in lower quantities in this subfraction compared to the two other subfractions, and a dominating peak with *m/z* 418.2946 (marked with a red arrow in Fig. 19) was suspected to be the compound that might cause the observed antibacterial activity, and thus selected for isolation and bioactivity confirmation. The same compound was also present in the active subfraction AM-24. The elemental composition of this compound was calculated to be $C_{25}H_{39}O_4N$, resulting in no hits

in the most common databases. In addition, two compounds with *m/z* 404.2788 (marked with blue arrows in Fig. 19) eluting in the active subfractions 23 and 24 were selected for isolation, since they were suspected to be isomers based on their different retention times and they might be bioactive. The calculated elemental composition of these compounds was $C_{24}H_{37}O_4N$, giving no hits in the most common databases.

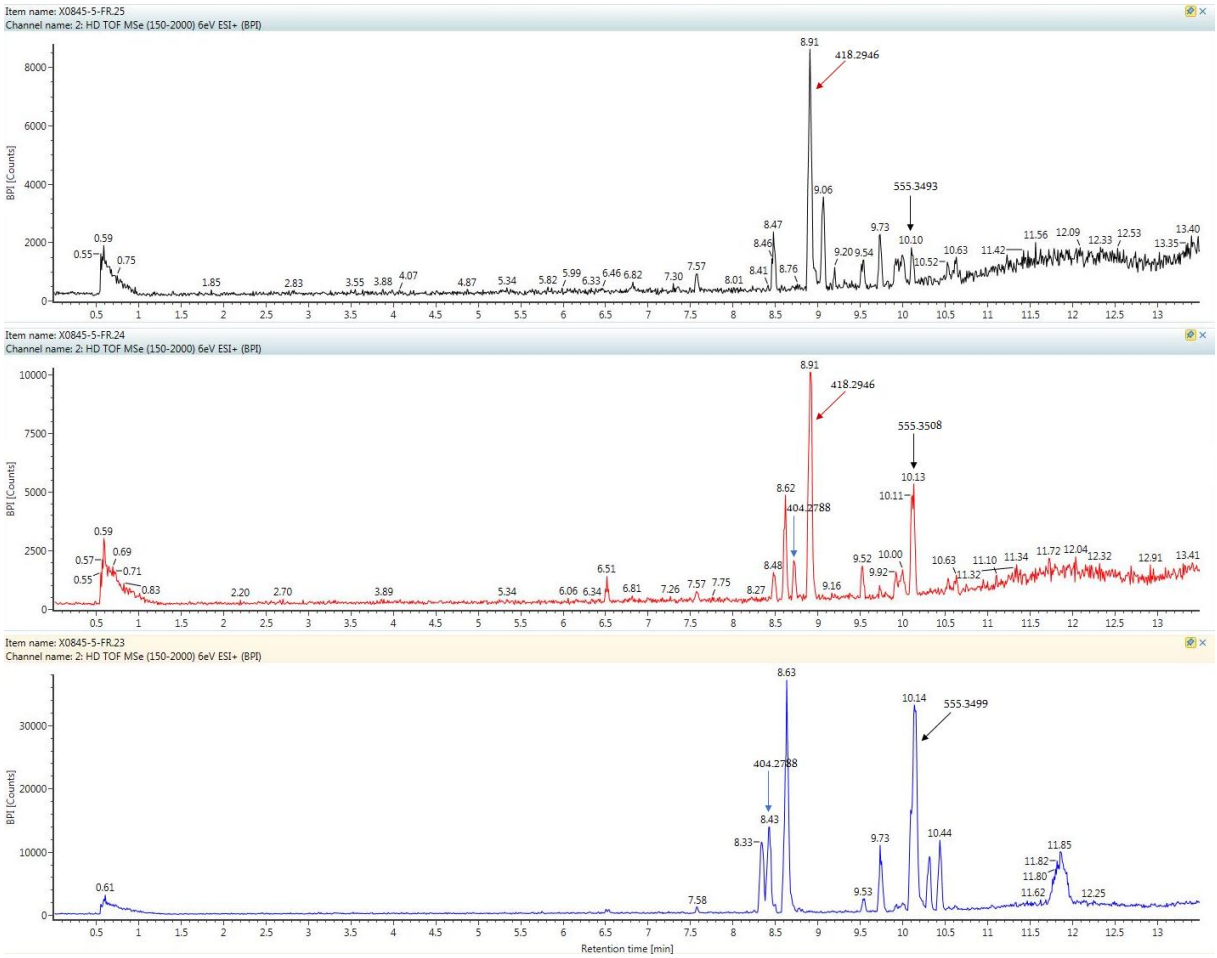

Figure 19: HR-MS BPI chromatogram of the active fraction subfractions AM-23, AM-24 and AM-25, in positive ESI mode. The fraction was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient of MilliQ water and Acetonitrile, both with 0.1% (v/v) FA, was used with a flow rate of 0.45 ml/min.

5.7 Preparative HPLC-MS isolation of target compounds

Target compounds were isolated from the bacterial strains using preparative HPLC-MS. The compounds selected for isolation were either suspected of being responsible for the observed bioactivity within the subfractions or had interesting chemical properties, such as halogenated compounds and isomers. The isolated compounds are summarised in Table 18, and Table 25 gives an overview of isolated compounds that are selected for further study.

5.7.1 Isolation of compounds from the bacterial strain TS

Preparative HPLC-MS purification was performed on flash fraction 5 from the extract of TS in order to isolate the target compounds in the active subfractions. The column X-Terra® Shield RP 18 was selected for the first round of HPLC separation and the fractions were collected by time triggered fractionation. The compounds from the bacterial strain TS selected for isolation based on the results from the antibacterial activity screening and dereplication, and the weight of the dried fractions after the first isolation are listed in Table 19. The BPI chromatogram from this isolation can be seen in Fig. 20.

Table 19: Isolated compounds from the bacterial strain TS and their dry weight after first purification.

Fraction	Weight (mg)
1. TS-25-398 and TS-25-386	1.6
2. TS-7-mix	29.1
3. TS-19-227	1.1
4. TS-4-652	3.3

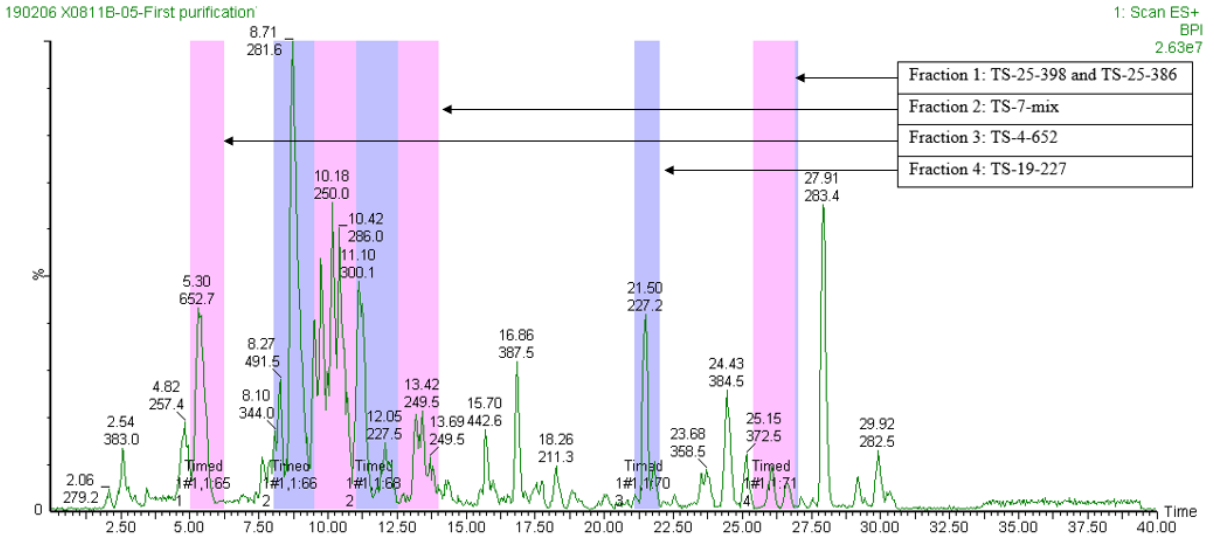

Figure 20: BPI chromatogram (ESI^+) from the first round of isolation of compounds considered responsible for the observed activity in MIC-assay from flash fraction 5 of the bacterial strain TS. The compounds were separated using a RP 18 column with an elution gradient of MilliQ water and ACN, both containing 0.1% (v/v) FA, and a flow rate of 6 mL/min (15% B to 100% B in 32 minutes). The fractions were collected by time triggered fractionation.

The fractions were further purified in a second round of preparative HPLC-MS in order to remove remaining impurities from the compounds. Purification was carried out using the fluoro-phenyl column and the fractions were collected by mass triggered fractionation. Fraction TS-4-652 contained a compound with *m/z* 265.2 (named TS-4-265) that was selected for further

purification from the first round of HPLC separation. The weight of the dried fractions is listed in Table 20, and the BPI chromatogram from this isolation can be seen in Fig. 21.

Table 20: Isolated compounds from the bacterial strain TS and their dry weight after second purification.

Fraction	Weight (mg)
TS-25-398*	0.3
TS-25-386*	0.2
TS-19-227	0.7
TS-4-652*	1.1
TS-4-265*	0.9

*indicates that they were excluded from further work

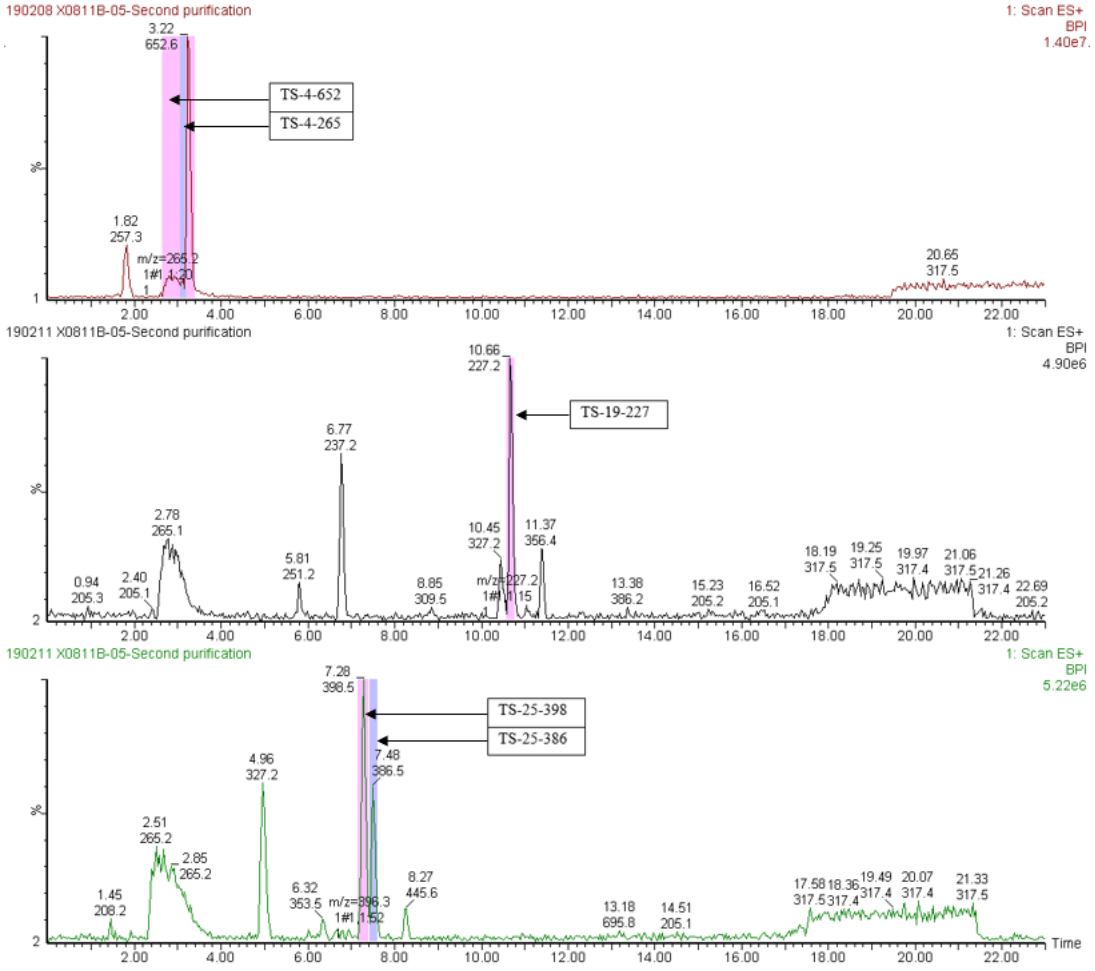

Figure 21: BPI chromatogram (ESI+) from the second purification of compounds considered responsible for the observed activity in MIC-assay from flash fraction 5 of the bacterial strain TS. The compounds were separated using a fluoro-phenyl column with an elution gradient of MilliQ water and ACN, both containing 0.1% (v/v) FA, and a flow rate of 6 mL/min. The fractions were collected by mass triggered fractionation.

The compounds TS-7-317 and TS-7-281 were isolated in one and two rounds of HPLC separation, respectively. These compounds were isolated from the fraction TS-7-mix as well as the flash fraction 5 using different columns and elution gradients in the first round of isolation (Table 18). However, the results of isolation from the fraction TS-7-mix is not shown in the

thesis since it follows the same principle as flash fraction 5 (Fig. 22). In the first round of isolation, the column Sunfire Shield RP 18 OBD™ was selected. The compound TS-7-317 was collected using time triggered fractionation, whereas the compound TS-7-281 was collected using mass triggered fractionation. Additionally, more of the compound TS-19-227 was isolated, as it was required for structure elucidation. The weight of the dried fractions is listed in Table 21, and the BPI chromatogram from this isolation can be seen in Fig. 22.

Table 21: Isolated compounds from the bacterial strain TS and their dry weight after first purification.

Fraction	Weight (mg)
1. TS-7-281	6.6
2. TS-7-317	5.6
3. TS-19-227	1.4

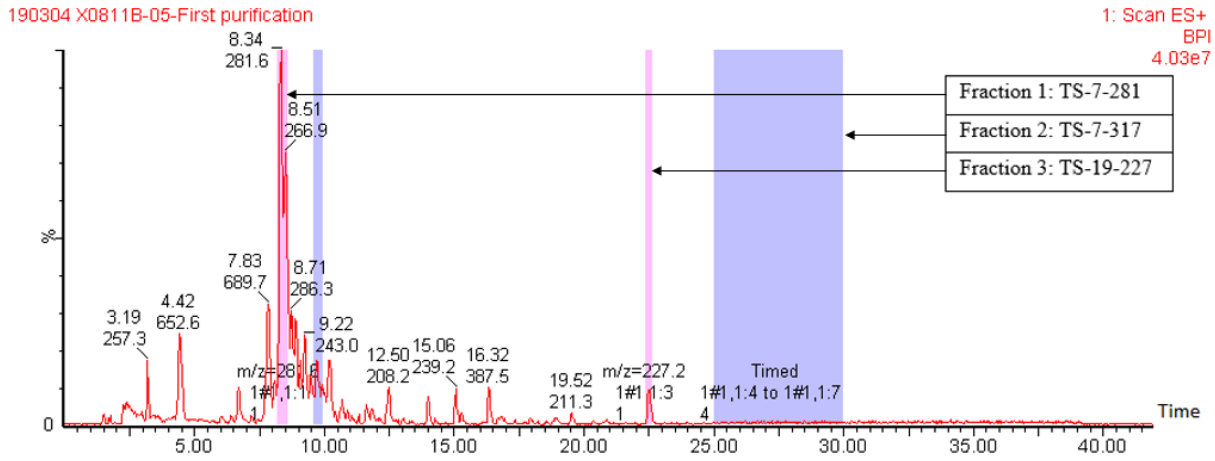

Figure 22: BPI chromatogram (ESI^+) from the first round of isolation of compounds considered responsible for the observed activity in MIC-assay of the fraction TS-7-mix and the compound TS-19-227. The compounds were isolated from flash fraction 5, using a RP 18 column with an elution gradient of MilliQ water and acetonitrile, both containing 0.1% (v/v) FA, and a flow rate of 6 mL/min (20% B to 100% B in 32 minutes). The fractions 1 and 3 were collected by mass triggered fractionation while the fraction 2 was collected by time triggered fractionation.

The fractions TS-7-281 and TS-19-227 were further purified in a second round of preparative HPLC-MS to obtain compounds of sufficient purity for NMR analysis. The fraction TS-7-281 contained a compound with *m/z* 558.5 (named TS-7-558) and some other compounds eluting from 10 to 13 minutes (named TS-7-rt10-13), which were selected for further purification from the first round of HPLC separation. To remove impurities from these fractions, a gradient elution of 20% B to 100% in 15 minutes on the fluoro-phenyl column was used. The fractions TS-7-281, TS-7-558 and TS-19-227 were collected using mass triggered fractionation, while the fraction TS-7-rt10-13 was collected using time triggered fractionation. The weight of the dried fractions is listed in Table 22, and the BPI chromatograms from these separations can be seen in Fig. 23.

Table 22: Isolated compounds from the bacterial strain TS and their dry weight after second purification.

Fraction	Weight (mg)
TS-19-227	0.5
TS-7-281	2.2
TS-7-558	1.8
TS-7-rt10-13	1.1

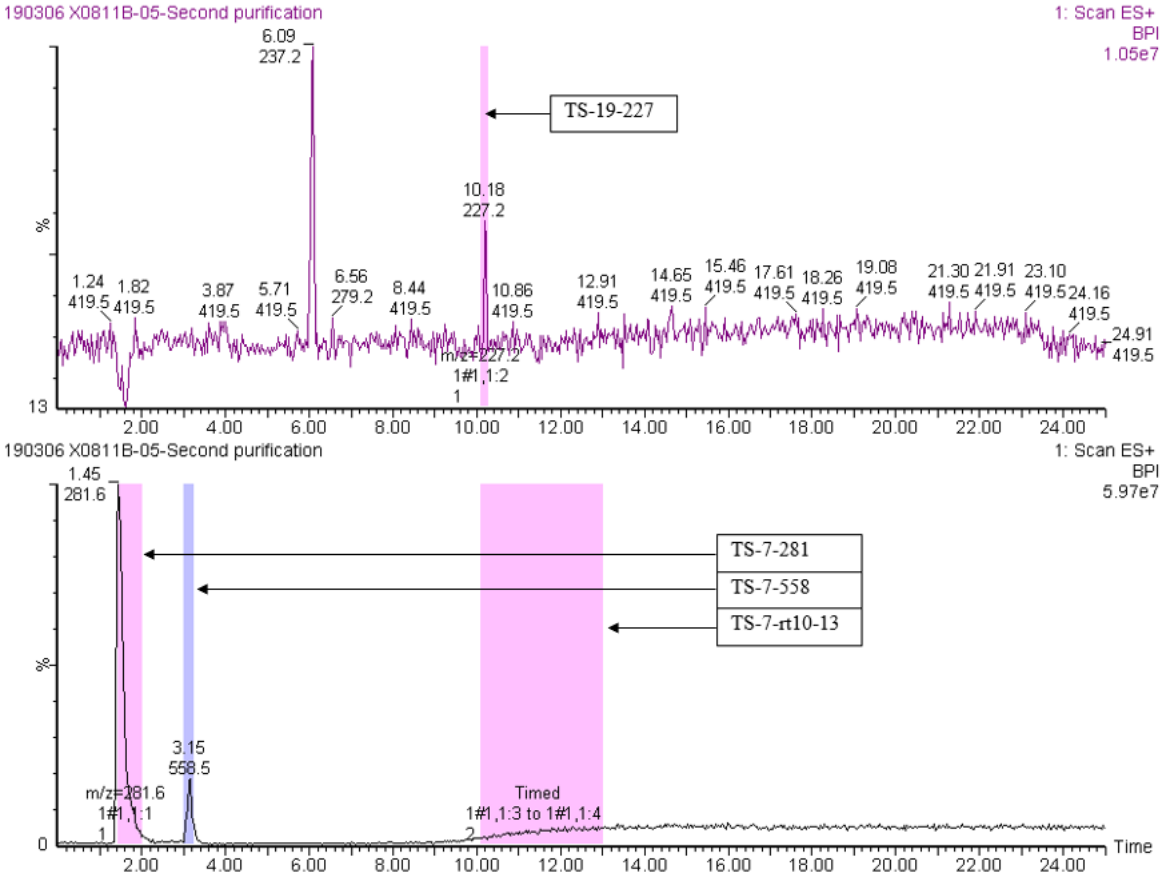

Figure 23: BPI chromatogram (ESI⁺) from the second purification of compounds considered responsible for the observed activity in MIC-assay. The compounds were separated using a fluoro-phenyl column with an elution gradient of MilliQ water and acetonitrile, both containing 0.1% (v/v) FA, and a flow rate of 6 mL/min (20% B to 100% B in 15 minutes). The fractions were collected by mass triggered fractionation while the fraction 2 was collected by time triggered fractionation.

5.7.2 Isolation of compounds from the bacterial strain AM

Three compounds with *m/z* 418.5, 404.5 and 404.5 were selected for isolation from the flash fraction 5 of the bacterial strain AM. The two compounds with same masses were eluting in both, subfractions 23 and 24 at different times, indicating it could be two compounds with different molecular structures (isomers). They were therefore isolated separately based on their retention times, and are named AM-23-404 and AM-24-404, respectively, in the thesis. The first isolation round was carried out using the column Sunfire Shield RP 18 OBD™ and the fractions were collected by mass triggered fractionation. The fractions were dried in the SpeedVac after the first round of isolation and prepared in the mobile phase gradient in which

they were eluting for the second round of isolation. The weight of the dried fractions is shown in Table 23, and the BPI chromatogram from this separation can be seen in Fig. 24.

Table 23: Isolated compounds from the bacterial strain AM and their dry weight after first purification.

Fraction	Weight (mg)
1. AM-23-404	2.4
2. AM-24-404	1.6
3. AM-25-418	1.5

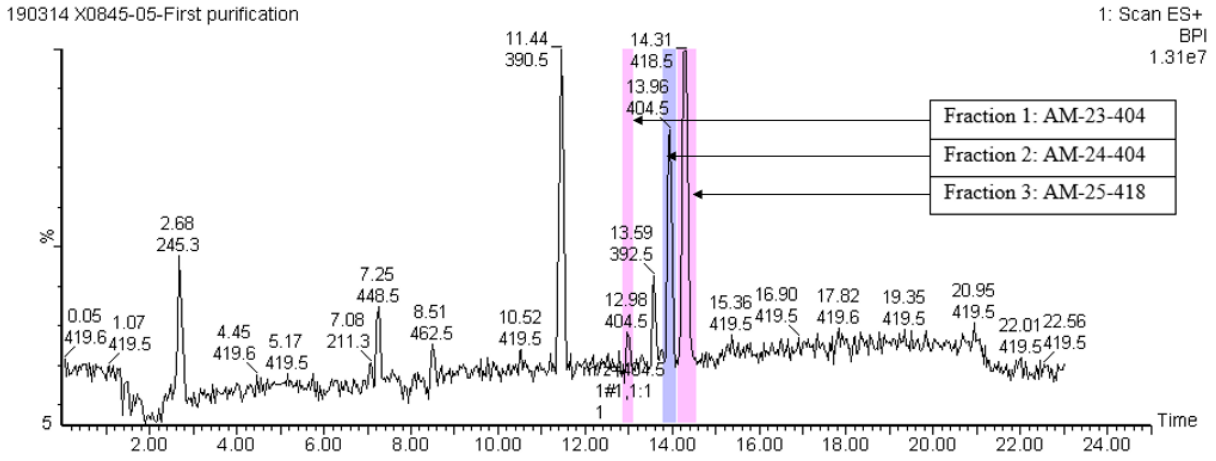

Figure 24: BPI chromatogram (ESI^+) from the first purification of compounds considered responsible for the observed activity from the bacterial strain AM. The compounds were separated using a RP 18 column with an elution gradient of MilliQ water and acetonitrile, both containing 0.1% (v/v) FA, and a flow rate of 6 mL/min (50% B to 100% B in 15 minutes. The fractions were collected by mass triggered fractionation. As seen in the chromatogram, the compound AM-23-404 is eluting earlier than the compound AM-24-404, indicating that the first mentioned compound has a more polar character.

The fractions were further purified in a second round of preparative HPLC-MS to obtain compounds of sufficient purity for structure elucidation by NMR. Purification was performed utilising the fluoro-phenyl column and the fractions were collected using mass triggered fractionation. The weight of the dried fractions is listed in Table 24, and the BPI chromatograms from these separations can be seen in Fig. 25.

Table 24: Isolated compounds from the bacterial strain AM and their dry weight after second purification.

Fraction	Weight (mg)
AM-23-404	1.1
AM-24-404	1.1
AM-25-418	1.0

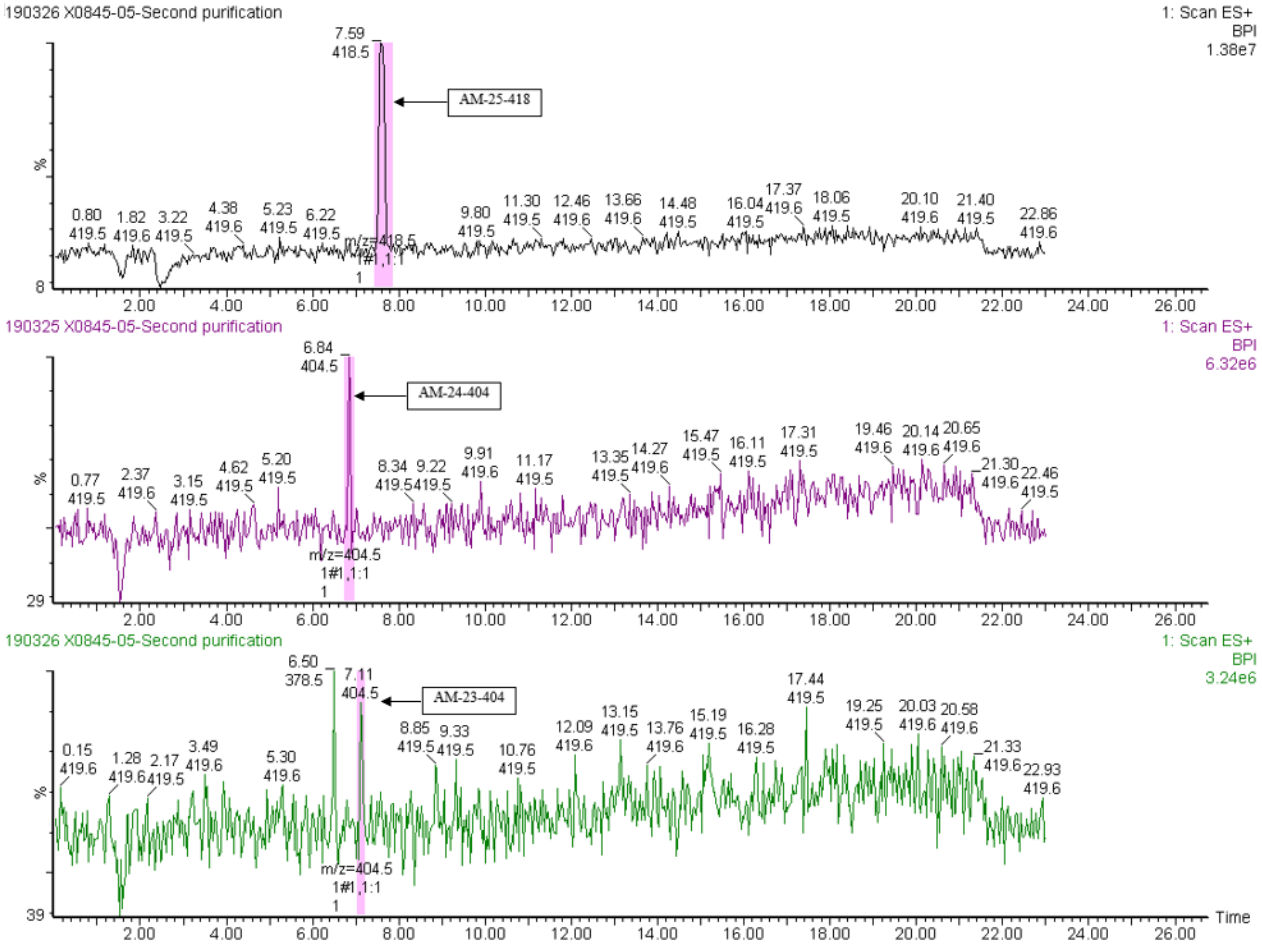

Figure 25: BPI chromatogram (ESI⁺) from the second purification round of compounds of interest from the bacterial strain AM. The compounds were separated using a fluoro-phenyl column with an elution gradient of MilliQ water and ACN, both containing 0.1% (v/v) FA, and a flow rate of 6 mL/min (50% B to 100% B in 15 minutes). The fractions were collected by mass triggered fractionation.

5.8 Bioactivity screening of the isolated target compounds

The isolated compounds were screened for antibacterial activity in a growth inhibition assay. The compounds were tested against the pathogenic bacterial strain *Streptococcus* type B in the concentrations 50, 10 and 5 µg/mL. Fraction TS-7-mix was active against the test strain at the highest and the lowest concentrations, while none of the other tested compounds displayed activity and were thus excluded from further work. The compounds most likely responsible for the observed activity in the fraction TS-7-mix were purified further, and the isolated compounds were re-tested for antibacterial activity against the same test strain at a final concentration of 50 µg/mL. The compound TS-7-281 was found to be responsible for the activity in this fraction as none of the other compounds were deemed active against the test strain. This compound as well as the compound TS-7-558, despite not being active, were submitted to structure elucidation by NMR. The compounds were also screened for anticancer activity against the human melanoma

celle line A2058 at a final concentration of 50 µg/mL, and the compound TS-7-281 was found to be active. The compound TS-7-317 was excluded from further work as it was not active and it was suspected of being a contaminant.

5.9 Structure elucidation of target compounds

Three isolated compounds from the bacterial strain TS and three isolated compounds from the bacterial strain AM were submitted to NMR in an attempt to achieve their full structures. The isolated compounds submitted to NMR, the criteria for why, their calculated elemental compositions and hits given in databases can be seen in Table 25. However, the chemical structure of the compounds was not achieved due to compound degradation and unforeseen downtime on the NMR at the end of project time, respectively.

Table 25: Overview of isolated compounds from fraction 5 of the bacterial strains TS and AM, that were submitted for structure elucidation with NMR and HR-MS analysis.

Fraction	Isolated compound (*m/z*)	Calculated elemental composition	Hits in databases	Amount for NMR (mg)	Criteria for structure elucidation and further investigation
TS-5	227.01	$C_{10}H_8N_2Cl_2$	- 2-Chloro-6-(4-chloro-1H-pyrrol-3-yl)-aniline - Benzenamine - Aminopyrrolnitrin - 2,3-Dichloro-6,7-dimethylquinoxaline	0.9	Di-halogenation and may be active in other bioassays
	281.6	-	-	1.7	Confirmed bioactivity
	558.6	-	-	1.3	Could be active in other bioassays
AM-5	418.5	$C_{25}H_{39}O_4N$	-	1.0	Believed to be bioactive
	404.5 (AM-23-404)	$C_{24}H_{37}O_4N$	-	1.1	Believed to be isomer and may be bioactive
	404.5 (AM-24-404)	$C_{24}H_{37}O_4N$	-	1.1	Believed to be isomer and may be bioactive

6.0 Discussion

6.1 Cultivation and extraction from the bacterial strains

Three marine bacteria (AM, PM and TS) were cultivated. AM and PM are classified under the class *Gammaproteobacteria* that belongs to the phylum Proteobacteria, the bacterial class that appears in most marine environments (104), whereas TS is a bacterium within the class *Alphaproteobacteria* that belongs to the phylum Proteobacteria as well, and is considered one of the greatest and most extensively studied groups within bacteria (105).

The bacteria were cultivated in different culture media with varying nutrient sources. It was attempted to make them grow in various culture media previously, and it was discovered that the media used were most suitable for these bacteria to grow in and produce bioactive secondary metabolites. After isolation, the bacteria have been cultivated in four different media, extracted *via* HP-20 solid phase extraction, flash-fractionated and tested in anticancer assays, antimicrobial assays and anti-inflammatory assays. That screening-campaign within the "Pharmasea"-project executed at Marbio was the basis for this master thesis, and the culture conditions that have shown activity or the highest activity were selected for the bacteria investigated in the present thesis. However, establishment of novel and further development of existing isolation and cultivation methods may increase the diversity of isolates, shorten the time of cultivation to attain appreciable cell mass for higher yields and production of secondary metabolites (106).

Extraction is the essential first step in NP drug discovery, as it is necessary to extract the coveted chemical compounds from raw materials for further separation and characterisation (107). The selection of an appropriate extraction method is driven by the amount and nature of the compounds to be isolated in the extraction process, as well as the matrix from which they are to be extracted (108). The compounds synthesised by bacteria are not known in advance, and thus it becomes a challenge to select a suitable extraction method in order to extract all compounds of interest. Solid-phase extraction with Diaion HP-20 resin and methanol is one of the standard extraction methods used at Marbio that has been proven to be suitable in previous studies (109).

It was cultivated 12 flasks, each containing 300 mL, of the bacterial strain PM, which gave an exctract yield of 18.33 g. TS and AM were cultivated in 21 flasks each, resulting in extraction yields of 17.75 and 6.05 g, respectively. As can be seen, PM had an overall higher extraction yield, whereas AM had the smallest yield of the three marine bacteria. Moreover, the yield per liter culture of PM was higher than of TS. However, the incubation period was longer for PM. This indicates that the crude extract's yield is dependent on the bacterial species and their incubation time. High extract weight however does not necessarily reflect high production of secreted secondary metabolites, as growth media components and primary metabolites may also be extracted. This was experienced in this thesis with the bacterial strain TS, where most of the compounds, including TS-4-652, TS-4-265 and TS-7-889, were found to be growth media components and not produced by the bacterial strain. Hence, the bacterium with the highest weight is not necessarily a better producer of secondary metabolites. Perhaps the bacterial strain AM produces low molecular weight secondary metabolites which are better adsorbed by the resin, whereas extracts of TS and PM contains higher amounts of growth media components.

6.2 Pre-fractionation of the extracts

The extracts from the bacterial strains were pre-fractionated into six fractions each prior to bioactivity screening. Only fraction 5 was found to be active in the bioactivity screening, and the yield obtained of this fraction was generally low. However, as can be seen in the results, fraction 5 from the extract of PM had a high yield since it was not completely dry. The fraction was placed in the speedvac for several days in an effort to remove organic solvent, and freezedried to remove the remaining water, but it was still some liquid left. Due to the low weight of the fraction from extracts of the other two bacterial strains, the potentially promising compounds of interest were present in too small quantities to carry out isolation, bioactivity confirmation and structure elucidation. Hence, new cultures were prepared to obtain sufficient material of the target compounds. When preparing new cultures, it is important that the same parameters are used for cultivation as the original culture, in order for the bacteria to produce the same active compounds of interest, as observed in this thesis. One of the major obstacles associated with the utilisation of MNPs in drug discovery are the difficulties in obtaining sufficient amounts of material pure enough for bioactivity confirmation and structure elucidation. Utilising marine bacteria is however an advantage in contrast to marine invertebrates, because if sufficient material is not obtained, it is feasible to prepare new cultures which mimic the same conditions in order to trigger the bacteria to produce the same

compounds. As for invertebrates, if a compound is obtained in small quantities and its availability is restricted or if the invertebrate is collected in regions difficult to access, re-supply becomes arduous.

6.3 Bioactivity screening of flash fractions

The flash fractions were analysed for antibacterial properties against six bacterial strains and screened for anticancer properties against the cancer cell line A2058. Cut-off values were used to assess whether a fraction was active or not, making it easy to quickly select which fractions to prioritise for further studies. The questionable fractions were treated as active and further analysed as these fractions may contain bioactive compounds of interest that will not be discovered if they are excluded from further investigation. Some compounds are of low potency and consequently show activity only at high concentrations, as observed with the flash fraction AM-5 which exhibited activity against MRSA at 100 and 50 µg/mL (Fig. 12 and 13), while the activity was lost at 25 µg/mL (Fig. 14). Hence, it is appropriate to carry out bioassays at various concentrations to perceive whether the compounds lose activity at lower concentrations.

No activity was reported against Gram-negative test strains in this project, which is consistent with previous results from Marbio, where activity against these bacteria are rarely observed. The need for novel antibiotics against Gram-negative bacteria is growing, as there is an immense problem with resistance observed for many of these organisms. Gram-negative bacterial infections may be difficult to treat due to the presence of porins and efflux pumps in their outer membrane, which can result in limited influx and increased outflux of antibiotics used during infections. Hence, it is important to discover novel antibiotics that are able to tackle the resistance of these bacteria (110). Cultivating the marine bacteria with Gram-negative bacteria could possibly trigger the production of secondary metabolites that are selectively active against these in a defence mechanism. However, the need for novel antibiotics against Gram-positive bacteria is also urgent, as there are several human pathogens amongst these organisms which are causing global resistance challenges, such as MRSA (111). Consequently, the research towards discovering novel antibiotics is becoming more important.

As can be seen in the results (Fig. 15), only fraction 5 from the extracts of PM and AM were deemed active against the A2058 cancer cell line. The activity was tested in a single high concentration (100 µg/mL), and the high activity observed in these fractions could be due to the high concentration. Substances that only display anticancer effects at very high

concentrations are unlikely to be sufficiently selective for cancer cells, because effects on other normal cells become increasingly more probable with higher concentrations. Hence, promising anticancer substances are usually highly potent and show activity at low concentrations. The fractions were however selected for further investigation in order to identify the compounds responsible for the observed activity and to find out if they were active at lower concentrations as well.

6.4 Re-fractionation of active flash fractions and bioactivity screening of subfractions

The active flash fractions were re-fractionated into 40 subfractions that were screened for antibacterial and anticancer activity. Subfractions are less complex than flash fractions, making it easier to identify compounds potentially responsible for the observed activity in bioassays for further investigation, as seen in this thesis. Moreover, by comparing active and inactive fractions, it was possible to select promising compounds for isolation and bioactivity confirmation. Compounds that were present in both active and inactive subfractions, in similar quantities, were excluded from further work. For instance, a compound with *m/z* 297.30 was observed in both the active subfraction TS-25 and the inactive subfraction TS-24, and was consequently excluded from further work. The subfractions were screened for antibacterial activity against the bacterial strains *E. coli* and *Streptococcus* type B. These bacterial strains were selected as representatives of Gram-negative and Gram-positive strains, respectively, because *E. coli* is the most-studied bacterial strain and *Streptococcus* type B is known as the most sensitive bacterial strain, according to the experience of Marbio, so if the subfractions are not active against this strain, they are probably not active against any other strains either. Although flash fraction 5 from the extract of TS did not display antibiotic activity against the pathogen *Streptococcus* type B, some of the subfractions did. This indicated that the active compounds either were present at low and not detectable concentrations or their effects were masked by interfering compounds. This illustrates the importance of re-fractionating the flash fractions into several less complex subfractions for further study.

Anticancer activity of the subfractions was evaluated against the human melanoma cancer cell line A2058, as this is the most sensitive cell line upon previous experience in Marbio. Activity was only observed in some subfractions from the bacterial strain PM at high concentration, thus the activity observed in flash fractions was confirmed to be due to the high concentration. RLs

may be responsible for the observed activity, as the same subfractions were active in antibacterial screening where RLs were observed and believed to be responsible for the activity.

It is important to mention that the re-fractionation process lacks the exact knowledge of tested concentration. It gives just qualitative information about the active fractions from which the active compounds may further be identified. Organic salts, lipids etc. may cause inspecific activity also due to synergic effects and especially if they are present in high concentrations. In such cases it can be a practical approach to dilute the re-fractionation to point out the most active fractions.

In the MS chromatogram of some active subfractions from the bacterial strains no compounds were detected, the compounds were however seen in the UV/Vis detector. This may indicate that the compounds have very strong chromophores, but are poorly ionised by ESI. These results show the importance of using a UV/VIS in series with a MS detector, as the two detectors provide complementary information.

6.5 Dereplication of active subfractions from bioactivity screening

In this thesis, 13 active subfractions (from three different bacterial strains) from the antibacterial activity screening were dereplicated using UHPLC-HR-MS to identify the compounds responsible for the observed bioactivity. An elemental composition was calculated for compounds believed to be active and database searches were performed. Dereplication proactively reduced the number of subfractions entering the isolation process, saving valuable time and resources (112). The active subfractions PM-19, PM-25, PM-27 and PM-29 contained known bioactive RLs, hence, further work with these subfractions was terminated. RLs were observed in the active subfractions AM-23, AM-24 and AM-25 as well, and to our knowledge they have not been reported from this bacterial strain previously (113). The subfraction TS-25 contained target compounds that appeared to be novel, so they were selected for isolation. The subfraction TS-19 contained a di-halogenated compound of interest that was selected for isolation. The subfractions AM-23 and AM-24 contained two compounds with the same masses but different retention times, that were suspected to be isomers and they were selected for isolation and structure elucidation to confirm this. The subfraction AM-25 contained a target compound that was selected for isolation as it was not identified in dereplication. In this thesis, it was possible to terminate further work with the fractions containing previously reported bioactive compounds (rhamnolipids) by doing dereplication prior to isolation.

Utilisation of UHPLC-HR-MS for dereplication brings challenges, such as the formation of adducts, that can affect the identification of compounds. As can be seen in the results, the RLs were found as sodium adducts, complying with previously isolated RLs in retention time and mass. Sodium adducts are frequently observed since the bacteria are grown in media containing filtered seawater.

6.6 Isolation and bioactivity confirmation of target compounds

The target compounds should be purified and isolated from the sample matrix in order to elucidate their chemical structures. To isolate target compounds from a sample consisting of several other compounds, it is important to select a suitable column and mobile phase gradient. Various columns can be used to obtain sufficient separation of target compounds and remove impurities utilising preparative HPLC-MS. The columns differ in packing material and may differ in particle size and column dimensions as well, giving rise to different retention of the compounds in a sample. In this project, three RP columns were utilised for HPLC separation (Fig. 26). The columns X-Terra® and SunFire^{TM} have a C18 hydrocarbon attached to their silica backbone, they however result in different separations due to differences in particle size and in silica-modifications. The Fluoro-Phenyl column incorporate multiple retention mechanisms, such as dispersion, shape selectivity, dipole, and pi-pi (π-π) interactions, making it a powerful tool for difficult separations, including those for small polar compounds and basic analytes. This column provides high selectivity by incorporating highly electronegative fluorine atoms on a phenyl ring (114). The compounds were isolated in only two rounds of HPLC separation in order to avoid losing much of the material, as at least 1 mg of material is required for NMR depending on structure complexity of the compounds. As seen in the results, the compound TS-7-317 was however isolated in only one round, since it was later suspected to be a contaminant based on the results seen in the BPI chromatograms from the second purification illustrated in Fig. 21.

By varying the use of columns in the first and second rounds of isolation in this thesis, target compounds of sufficient purity were obtained. The columns were selected based on prior experience with separation of target compounds, where a RP C18 column normally is used in the first round of isolation as a wide range of compounds are isolated with this column, while in the second round of isolation a more selective column is used to selectively isolate the target compound and get rid of impurities.

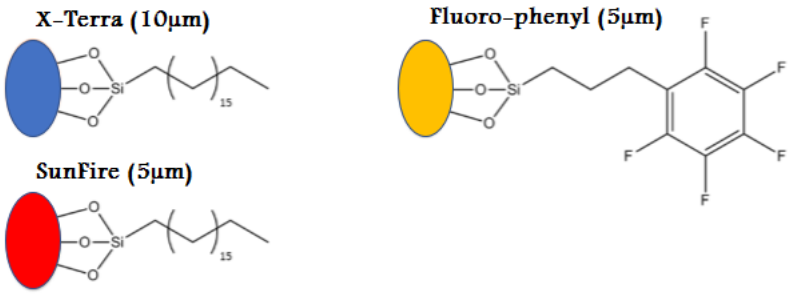

Figure 26: The column material of the three different RP columns utilised in this work. The circles with different colours represent the various silica backbone of the columns (115).

Compounds should be re-dissolved in a suitable solvent for the second round of isolation. However, this might become a challenge since the nature of the isolated compounds are unknown. Nevertheless, the retention times of the compounds provide an indication of their hydrophobicity and hydrophilicity, as the polar compounds elute earlier than the non-polar compounds in a RP C18 column used in the first round of isolation. Methanol is a preferred solvent in order to re-dissolve NPs, as it should dissolve most of these compounds. However, a ''trial-and-error'' approach should be carried out if the compounds are not soluble in methanol (74). As experienced in this thesis, the compounds TS-19-227, AM-23-404, AM-24-404 and AM-25-418 were found difficult to re-dissolve for the second round of isolation, and they were tested in several solvents in an effort to dissolve them. After all, the compound TS-19-227 was found to dissolve best in tetrahydrofuran, acetonitrile and MilliQ water, whereas the other compounds were found to dissolve best in the mobile phase gradient in which they were eluting in the first round of isolation.

Some interfering compounds may lead to false positive results, so there is no assurance that isolated compounds are responsible for the observed biological activity detected in the screening. It is therefore, a possible strategy to screen the isolated compounds for bioactivity prior to NMR analysis if sufficient material is obtained, in order to confirm their activities with certainty. Importantly, by confirming bioactivity before submitting compounds suspected to be active for structure elucidation with NMR, time and resources where saved as compounds found to not be active were excluded from further study. For instance, the two compounds, TS-25-398 and TS-25-386, which were suspected to be responsible for the observed activity in subfraction 25, were found not to be active. They were tested for bioactivity in a high concentration (50µg/mL) against one of the most sensitive bacterial strains, and as they were not active it was decided to exclude them from further study. It might be that a potent compound present in a very low concentration that actually is responsible for the observed bioactivity. The

other compounds present in this fraction should be isolated and screened for bioactivity in order to confirm this. This is however a time- and resource consuming process to carry out in this thesis. The activity may possibly also be due to a synergic effect from several compounds present in the fraction.

For fraction TS-7 it was difficult to identify the compounds responsible for the bioactivity, and as isolation is time and resource consuming, it was decided to isolate several compounds of interest in a fraction named TS-7-mix (Fig. 20), and screen them for antibacterial activity to confirm potential activity before any further isolation of the compounds. The fraction was found to be active against the bacterial strain *Streptococcus* type B and was hence dereplicated to identify the compounds most likely responsible for the observed activity. By comparing this subfraction with that of the medium used in the cultivation of TS, in dereplication, it was possible to avoid selecting growth media components for isolation and isolate the compound responsible for the observed bioactivity. The compound TS-7-281 was found to be responsible for the bioactivity, and it was active in both antibacterial and anticancer assays. Therefore, it was submitted to NMR as it was not identified in dereplication, and appeared thus to be novel. The BPI chromatogram of the compound can be seen in Appendix, see A1. The compound TS-7-558 was submitted to NMR as well, despite not being active, as it may be active in other bioassays or its chemical properties may be of interest.

A di-halogenated compound was observed in the inactive subfraction TS-19. The compound was found interesting despite not being active in the bioassays performed in this thesis, as it may be active in other bioassays and the presence of halogens in pharmaceuticals is known to affect their activity by altering their electronic and steric properties, improving their membrane permeability, slowing their catabolism and improving their binding affinity with their targets (116). According to HR-MS, this compound is probably an aminopyrrolnitrin, and if so, this is the first time an aminopyrrolnitrin is isolated from this bacterial strain. Aminopyrrolnitrin is a precursor of pyrrolnitrin which is a previously reported NP produced by the bacterial strain *Pseudomonas* sp., and has known antifungal activity (117). The compound was however selected for isolation and structure elucidation with NMR, as it was not possible to identify it with high certainty in dereplication. This compound was submitted to NMR before performing further bioassays to investigate its activity, as only a small quantity of the compound was obtained after isolation and as much as possible was required for NMR.

The compounds AM-23-404 and AM-24-404 were as mentioned selected for isolation as they were believed to be isomers. Isomers are of interest in NP drug research, as they may differ in

their pharmacokinetic and pharmacodynamic properties, and consequently result in different bioactivities. Moreover, isolation of isomers which are strictly related compounds is highly appreciated, as it can be used to assess structure-activity relationships (SAR) (118). The compound AM-25-418 was believed to be responsible for the bioactivity in subfraction AM-25 and was selected for isolation to confirm this. These compounds were submitted to NMR before investigating their activities due to restricted time with the thesis and to ensure the achievement of full structures prior to further investigation.

In the active subfractions TS-3, TS-26 and TS-27, it was not detected any compounds of interest for isolation. The compounds eluting in the beginning of the gradient in a RP-HPLC separation are highly hydrophilic, such as sugars or amino acids, while the compounds eluting in the end of the gradient are in general highly lipophilic such as phospholipids. Promising bioactive compounds to be used as pharmaceuticals have generally intermediate lipophilicity and are thus eluting in the middle of the gradient. Highly hydrophilic compounds have low bioavailability as they cannot cross biological membranes, and highly lipophilic compounds can accumulate in the bloodstream as they do not dissolve well. Therefore, a desirable property of an effective pharmaceutical is a balanced hydrophilic/lipophilic character. However, it is not proven that the compounds responsible for the observed activity in these fractions are sugars, amino acids or phospholipids. Several growth media components were observed in the fractions that possibly could be responsible for the bioactivity. Observed activity may also be due to a synergic effect of several compounds in the fractions. It is nonetheless not possible to predict the respective compounds without further investigation, which is time consuming and not appropriate in this work. As experienced in this work, identifying compounds causing observed bioactivity within fractions is one of the major challenges in NP drug discovery, as most of the time, the compounds suspected of being active are found to be inactive.

6.7 Structure elucidation of target compounds by NMR

The bioactive and di-halogenated compounds were prioritised for NMR analysis, since they were the most promising and interesting among the isolated compounds. However, only the di-halogenated compound was analysed by NMR. No NMR analysis was conducted for the other compounds, due to unforeseen downtime on the NMR at the end of project time. Also due to the time limit we had, we were not able to conduct further analysis. Therefore, no NMR data is available for these. This will be part of further work.

The di-halogenated compound TS-19-227 was not sufficiently pure to enable elucidation of the structure. NMR spectra of the compound showed only weak signals and purity of the compound was roughly 10-15%, implicating presence of high impurities which unable characterisation of the target compound. Therefore, it was difficult to interpret the spectra. However, NMR analysis could confirm the presence of two halogens in the structure. To our knowlegde, this is the first time a di-halogenated compound with potential bioactivity is isolated from the bacterial strain TS. The weak signals were suspected of being a result of poor solubility in DMSO, there was however no attempt to dissolve it in other solutions, as more compound in pure form was needed to conduct further NMR analysis. Mass spectrometry may allows conclusions about the compound of interest when there is not enough sample for an NMR analysis. The fragmentation pattern of a molecule in mass spectrometry can provide useful clues about the compounds structure and identity, it however does not prove the structure and thus, a full synthesis and characterisation would be needed as final proof (119). The compound was run in positive and negative ESI mode, and the respective MS chromatograms can be seen in Appendix, see A2. Most of the compounds detected in positive ESI mode were found to be di-chlorinated compounds based on their mass spectra, and were therefore suspected of being degradation products of the target compound which was not detected in the chromatogram. It is known that extracts from any sort of organism may consist of highly labile compounds which may decompose at any stage in the purification process or storage, and this might apply to this compound. Factors that may lead to degradation of compounds include heat, light, air, pH and solvents.

The purification process of the target compound was repeated, and it was run in HR-MS again to confirm wheter if it was degraded. The target compound was not detected, another di-halogenated compound with *m/z* 236.99, named TS-236, was however seen in the chromatogram (Appendix; A3). The calucalated elemental composition of the compound was $C_{11}H_6N_2Cl_2$, giving several hits in the databases. All the suggested compounds had a cyano group in common, but their position of the two chlorine atoms was different. One of the suggestens was the compound fenpicionil, and it seems to be a degradation product of the target compound that is suspected of being aminopyrrolnitrin, based on its structure and as it was not detected in flash fraction 5. The compounds are depicted in Fig. 27, and as can be seen, the primary amine of aminopyrrolnitrin is probably oxidised to form a nitrile (fenpicionil) in several stages. Primary amines are quite reactive because of their basicity as well as their nucleophilicity, so they are involved in oxidation and hydroxylation reactions in many chemical

and biological systems. Aromatic amines are additionaly extremely reactive in electrophilic aromatic substitution. The mechanism for the occurred reaction is difficult to predict without further investigation, as the synthesis of nitriles from primary amines can take place in several ways. It might be that the target compound is involved in a chemical reaction with one or several of the solvent used in the re-fractionation and isolation process, such as acetonitrile and formic acid, DMSO and methanol were not options as these were used in the flash fractionation and to dissolve the flash fractions without affecting the compound since we have found 227 but no 236 within the MS profile of our flash fractions, indicating that to that step of the preparation no degradation has taken place. The target compound might also be involved in chemical reactions with other compounds present in the fraction. A possible reaction mechanism might be that the primary amine is involved in a reaction with formic acid or other unknown compounds within the fraction, to form an amide that is further dehydrated by acetonitrile into a nitrile (120). Another possible mechanism behind this reaction might be transition metal catalysed cyanation of aryl halides (121). However, the additional purification (employing the same method) with subsequent UHPLC-MS/MS analysis proved that the purification itself worked and the purity would have been sufficient for NMR. The compound itself is unstable and the test of different solvents to dissolve the compound for NMR might even have increased its decomposition.

Subfraction TS-19 was analysed with HR-MS to investigate if the target compound was present, and to verify whether it was decomposed or not. The respective BPI chromatogram can be seen in Appendix, see A4. As can be seen, the compound was detected. However, the compound which was suspected to be a product of decomposition was also detected. Moreover, several other compounds with similar structures as the target compound were detected, indicating decomposition had taken place. This proves that the target compound gradually starts to decompose in the re-fractionation process. The structures given in Fig. 27 are of course hits generated by MS data, however the results of the Chemspider search and the mass difference between TS-227 and TS-236 complie with the conversion of an amide into a nitrile.

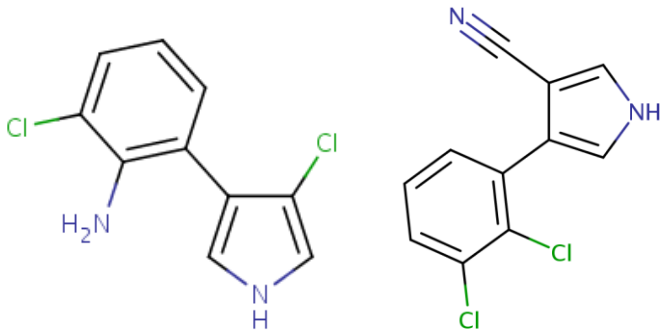

Figure 27: Structures of Aminopyrrolnitrin (left) and Fenpicionil (right).

7.0 Conclusion

Six compounds were isolated from two different bacterial strains using the bioassay guided isolation approach, and their structures were attempted elucidated by NMR spectroscopy. One bioactive compound was isolated from the bacterial strain TS. The compound was active against the bacterial strain *Streptococcus* type B and the melanoma cell line A2058, at a final concentration of 50 µg/mL. Moreover, a di-halogenated compound was also isolated from this bacterial strain, and it was attempted characterised by NMR without results. The compound was degraded, and it was therefore difficult to interpret the NMR spectra. RLs were observed in fractions from the bacterial strain AM for the first time.

The results from this work illustrate

- That bioprospecting of marine bacteria enables isolation of bioactive secondary metabolites, and previously reported compounds in novel species.
- The importance of dereplication to terminate samples that should not be prioritised in a bioprospecting process.
- The importance of isolating compounds in several preparative HPLC(-MS-DAD) rounds in order to obtain compounds of sufficient purity.
- That compounds of interest might decompose during the isolation process. Here the suitabillity of HPLC-MS to check purity and stabillity of the compounds of interest has been proven.

8.0 Outlook

In this work process, several compounds were isolated and screened for bioactivity to confirm their activities. Six isolated compounds (Table 25) were found interesting and submitted to NMR spectroscopy for identification. NMR analaysis was only performed for the compound TS-19-227, the structure of the compound was not elucidated since it was degraded. The compound TS-236 was found to be a degradation product of this compound, and it will be analysed by NMR spectroscopy, as it can give an indication of what may have contributed to degradation of the target compound. The target compound will be attempted structure elucidated at a later stage, and screened for bioactivity in several assays to characterise its potential activity. The compound TS-7-281 was found to be active in antibacterial and anticancer assays at a final concentration of 50 µg/mL, further work will be done on determinig the IC_{50} value of the compound to asses its potency. The chemical structure of this compound was not achieved during this work, but NMR analysis will be performed in future work. NMR analysis will be performed for the compounds AM-25-418, AM-24-404 and AM-23-404 as well. These compounds will also be screened for bioactivity in several assays to test for their bioactivities.

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10.0 Appendix

A1: UHPLC-QToF-MS of the compound TS-7-281

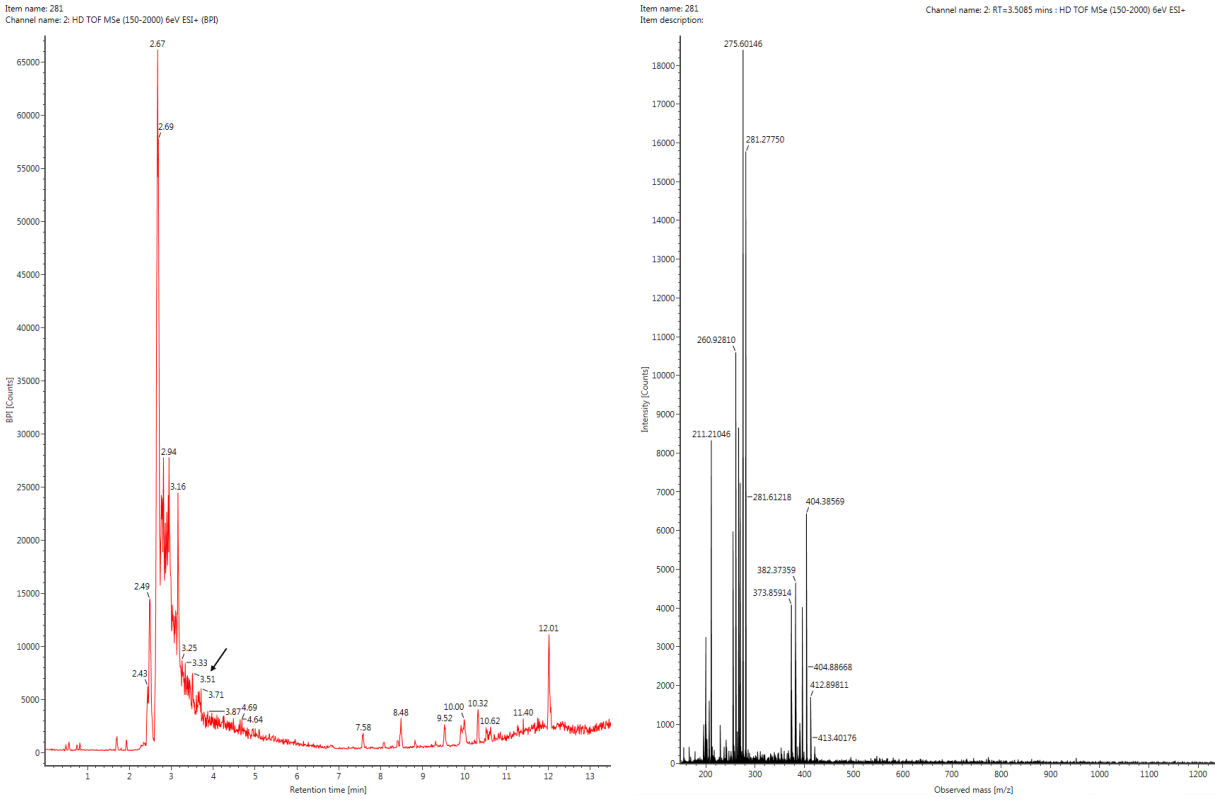

Figure 28: Left) HR-MS BPI chromatogram (ESI⁺) of fraction TS-7-281 after the first round of isolation. The fraction was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient og MilliQ and Acetonitrile, both with 0.1% FA was used with a flow rate of 0.45 ml/min. Right) The mass spectrum of the compound TS-7-281. No elemental composition was calculated for this compound.

A2: UHPLC-QToF-MS of the compound TS-19-227 after NMR analysis

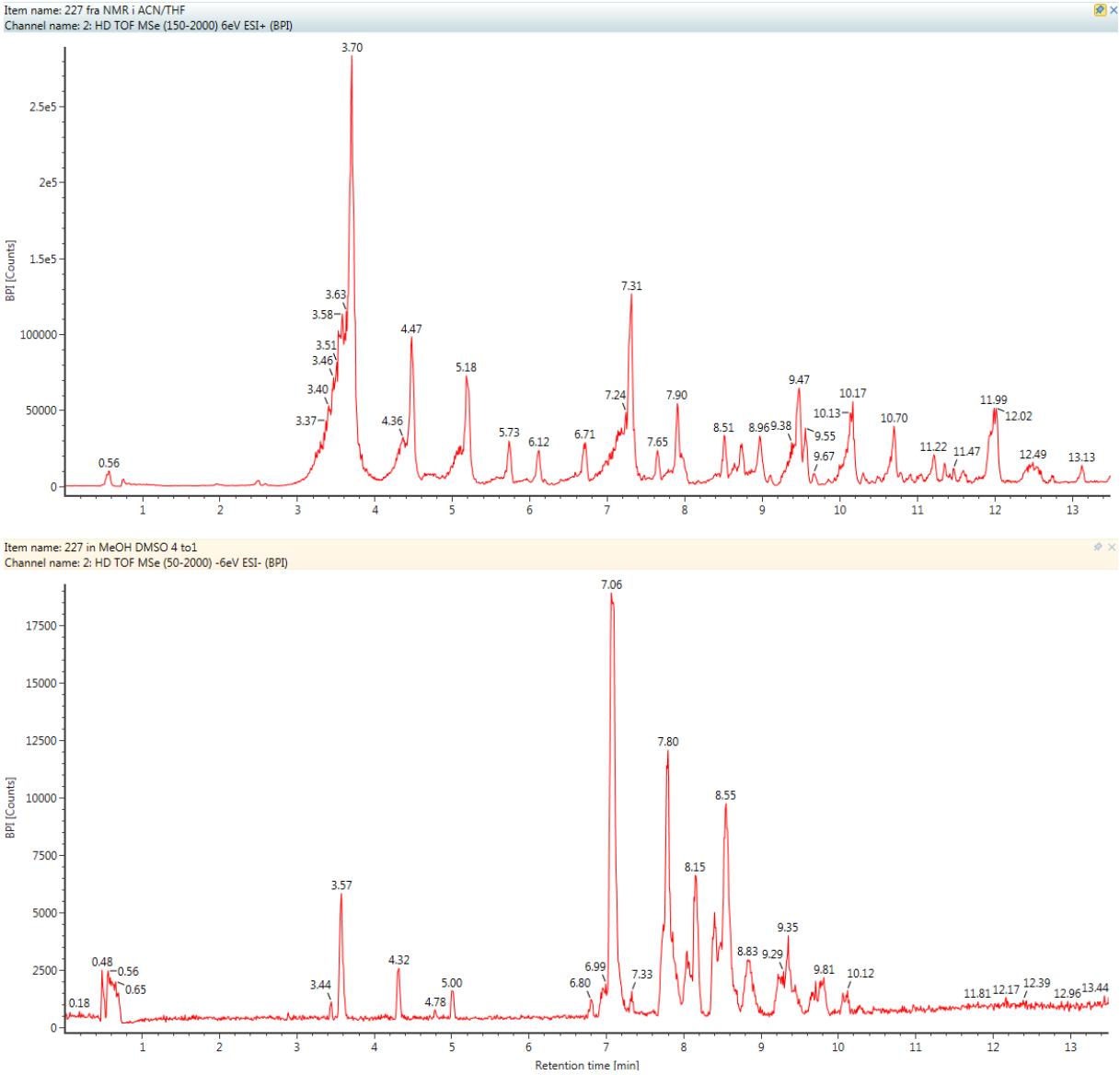

Figure 29: HR-MS BPI chromatograms (ESI^+ and ESI^-) of the di-chlorinated compound 227. The compound was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient og MilliQ and Acetonitrile, both with 0.1% FA was used with a flow rate of 0.45 ml/min. The chromatograms show that the target compound is not detected and most of the compounds detected in positive ESI mode were di-chlorinated, such as the compounds with retention times 3.70, 4.47, 5.18, 5.73 and 7.31. This could imply that the target compound is degraded during the isolation process or it could be unstable in DMSO which was used for NMR analysis.

A3: UHPLC-QToF-MS of the compound TS-19-227 after a new attempt of isolation

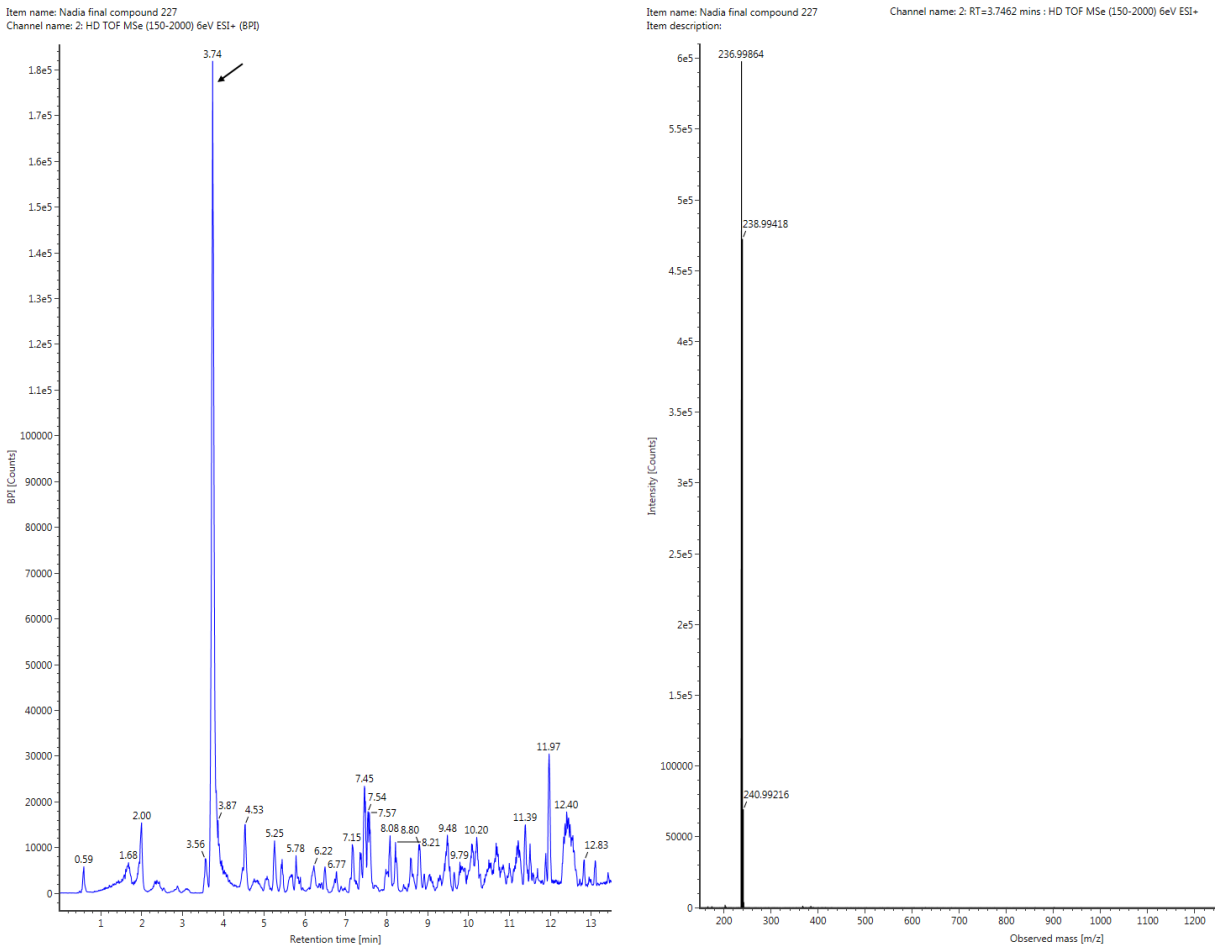

Figure 30: Left) HR-MS BPI chromatogram (ESI⁺) of the di-chlorinated compound 227. The compound was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient og MilliQ and Acetonitrile, both with 0.1% FA was used with a flow rate of 0.45 ml/min. The chromatogram show that the compound 227 is not detected, another compound with m/z 236.99 was however detected as seen. Right) The mass spectrum of the compound 236.99. As can be seen the compound is di-chlorinated, and this compound was not found in the flash fraction 5. Therefore, it was suspected of being a degradation product of the compound 227.

A4: UHPLC-QToF-MS of subfraction TS-19

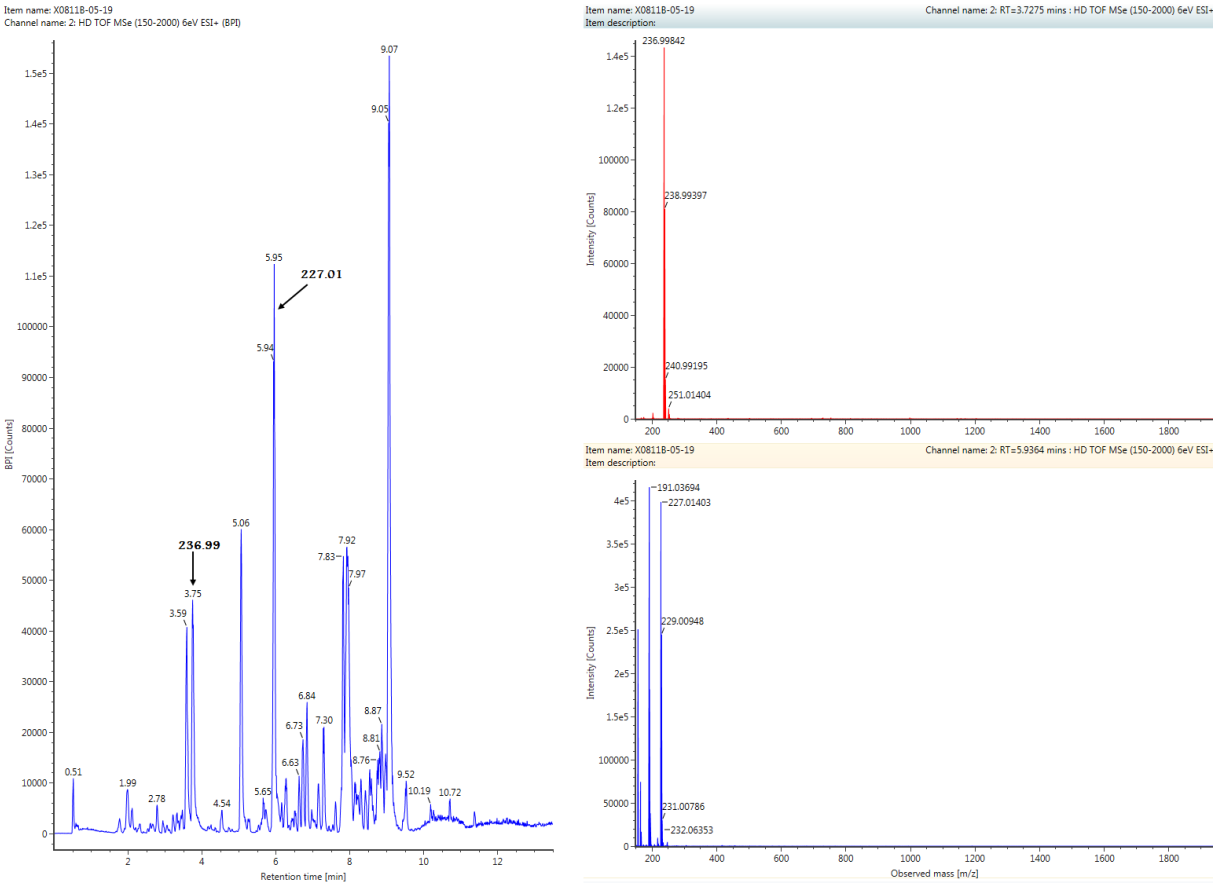

Figure 31: Left) HR-MS BPI chromatogram (ESI⁺) of subfraction TS-19. The subfraction was injected into a Waters Aquity UPLC® (2.1x100 mm, 1.7 µm) column, and a mobile phase gradient og MilliQ and Acetonitrile, both with 0.1% FA was used with a flow rate of 0.45 ml/min. The chromatogram show that the compound 227 is detected, and the compound TS-236, suspected of being a degradation product of this compound is also detected. Right) The mass spectra of the compound TS-19-227 and TS-236. This proves that the compound TS-236 is a degradation product of the target compound as the compound was not detected in flash fraction 5, and their structures are similar based on MS data.